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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: VECTORS AND METHODS FOR GENE TRANSFER TO CELLS
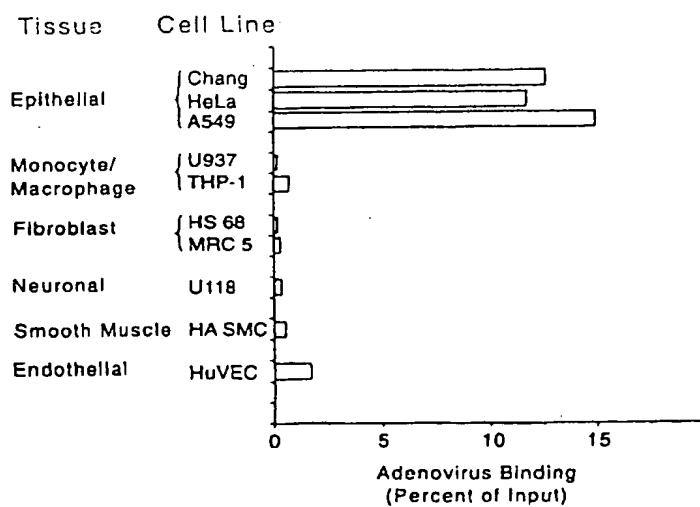
(57) Abstract

The present invention provides a chimeric adenovirus coat protein, which differs from the wild-type coat protein by the introduction of a nonnative amino acid sequence. Such a chimeric adenovirus coat protein according to the invention is able to direct entry into cells of a vector comprising the coat protein that is more efficient than entry into cells of a vector that is identical except for comprising a wild-type adenovirus coat protein rather than the chimeric adenovirus coat protein. The chimeric coat protein preferably is a fiber, hexon, or penton protein. The present invention also provides an adenoviral vector that comprises the chimeric adenovirus coat protein, as well as methods of constructing and using such a vector.

VECTORS AND METHODS FOR GENE TRANSFER TO CELLS

TECHNICAL FIELD OF THE INVENTION

The present invention pertains to a chimeric
adenovirus coat protein which is able to direct entry
into cells of a vector comprising the coat protein that
is more efficient than a similar vector having a wild-
type adenovirus coat protein. Such a chimeric coat
protein is a fiber, hexon, or penton protein. The
present invention also pertains to a recombinant vector
comprising such a chimeric adenoviral coat protein, and
to methods of constructing and using such a vector.

BACKGROUND OF THE INVENTION

Adenoviruses belong to the family Adenoviridae,
which is divided into two genera, namely Mastadenovirus
and Aviadenovirus. Adenoviruses are nonenveloped,
regular icosahedrons of about 65 to 80 nanometers in
diameter (Horne et al., J. Mol. Biol., 1, 84-86 (1959)).
The adenoviral capsid is composed of 252 capsomeres of
which 240 are hexons and 12 are pentons (Ginsberg et al.,
Virology, 28, 782-783 (1966)). The hexons and pentons
are derived from three different viral polypeptides
(Maizel et al., Virology, 36, 115-125 (1968); Weber et
al., Virology, 76, 709-724 (1977)). The hexon comprises
three identical polypeptides of 967 amino acids each,
namely polypeptide II (Roberts et al., Science, 232,
1148-1151 (1986)). The penton contains a penton base,
which is bound to the capsid, and a fiber, which is
noncovalently bound to and projects from the penton base.
The fiber protein comprises three identical polypeptides
of 582 amino acids each, namely polypeptide IV. The
adenovirus serotype 2 (Ad2) penton base protein is a
ring-shaped complex composed of five identical protein
subunits of 571 amino acids each, namely polypeptide III
(Boudin et al., Virology, 92, 125-138 (1979)). Proteins
IX, VI, and IIIa are also present in the adenoviral coat

and are thought to stabilize the viral capsid (Stewart et al., Cell, 67, 145-154 (1991); Stewart et al., EMBO J., 12(7), 2589-2599 (1993)).

5   Once an adenovirus attaches to a cell, it undergoes receptor-mediated internalization into clathrin-coated endocytic vesicles of the cell (Svensson et al., J. Virol., 51, 687-694 (1984); Chardonnet et al., Virology, 40, 462-477 (1970)). Virions entering the cell undergo a stepwise disassembly in which many of the viral
10   structural proteins are shed (Greber et al., Cell, 75, 477-486 (1993)). During the uncoating process, the viral particles cause disruption of the cell endosome by a pH-dependent mechanism (Fitzgerald et al., Cell, 32, 607-617 (1983)), which is still poorly understood. The viral
15   particles are then transported to the nuclear pore complex of the cell (Dales et al., Virology, 56, 465-483 (1973)), where the viral genome enters the nucleus, thus initiating infection.

An adenovirus uses two separate cellular receptors,
20   both of which must be present, to efficiently attach to and infect a cell (Wickham et al., Cell, 73, 309-319 (1993)). First, the Ad2 fiber protein attaches the virus to a cell by binding to an as yet unidentified receptor. Then, the penton base binds to α_v integrins, which are a
25   family of a heterodimeric cell-surface receptors that mediate cellular adhesion to the extracellular matrix molecules, as well as other molecules (Hynes, Cell, 69, 11-25 (1992)).

The fiber protein is a trimer (Devaux et al., J.
30   Molec. Biol., 215, 567-588 (1990)) consisting of a tail, a shaft, and a knob. The fiber shaft region is composed of repeating 15 amino acid motifs, which are believed to form two alternating β-strands and β-bends (Green et al., EMBO J., 2, 1357-1365 (1983)). The overall length of the
35   fiber shaft region and the number of 15 amino-acid repeats differ between adenoviral serotypes. For example, the Ad2 fiber shaft is 37 nanometers long and

contains 22 repeats, whereas the Ad3 fiber is 11
nanometers long and contains 6 repeats. The receptor
binding domain of the fiber protein is localized in the
knob region encoded by the last 200 amino acids of the
5   protein (Henry et al., J. Virology, 68(3), 5239-5246
(1994)). The regions necessary for trimerization are
also located in the knob region of the protein (Henry et
al. (1994), supra). A deletion mutant lacking the last
40 amino acids does not trimerize and also does not bind
10 to penton base (Novelli et al., Virology, 185, 365-376
(1991)). Thus, trimerization of the fiber protein is
necessary for penton base binding. Nuclear localization
signals that direct the protein to the nucleus to form
viral particles following its synthesis in the cytoplasm
15 are located in the N-terminal region of the protein
(Novelli et al. (1991), supra). The fiber, together with
the hexon, are the main antigenic determinants of the
virus and also determine the serotype specificity of the
virus (Watson et al., J. Gen. Virol., 69, 525-535
20 (1988)).

Recombinant adenoviral vectors have been used for
the cell-targeted transfer of one or more recombinant
genes to diseased cells or tissue in need of treatment.
Such vectors are characterized by the advantage of not
25 requiring host cell proliferation for expression of
adenoviral proteins (Horwitz et al., In Virology, Raven
Press, New York, vol. 2, pp. 1679-1721 (1990); and
Berkner, BioTechniques, 6, 616 (1988)). Moreover, if the
targeted tissue for somatic gene therapy is the lung,
30 these vectors have the added advantage of being normally
trophic for the respiratory epithelium (Straus, In
Adenoviruses, Plenan Press, New York, pp. 451-496
(1984)).

Other advantages of adenoviruses as potential
35 vectors for human gene therapy are: (i) recombination is
rarely observed with use of such vectors; (ii) there are
no known associations of human malignancies with

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adenoviral infections despite common human infection with
adenoviruses; (iii) the adenoviral genome (which is a
linear, double-stranded DNA) can be manipulated to
accommodate foreign genes that range in size; (iv) an
5   adenoviral vector does not insert its DNA into the
chromosome of a cell, so its effect is impermanent and
unlikely to interfere with the cell's normal function;
(v) the adenovirus can infect non-dividing or terminally
differentiated cells, such as cells in the brain and
10   lungs; and (vi) live adenovirus, having as an essential
characteristic the ability to replicate, has been safely
used as a human vaccine (Horwitz et al. (1990), supra;
Berkner et al. (1988), supra; Straus et al. (1984),
supra; Chanock et al., JAMA, 195, 151 (1966); Haj-Ahmad
15   et al., J. Virol., 57, 267 (1986); and Ballay et al.,
EMBO, 4, 3861 (1985); PCT patent application WO
94/17832).

A drawback to adenovirus-mediated gene therapy is
that significant decreases in gene expression are
20   observed after two weeks following administration of the
vector. In many therapeutic applications, the loss of
expression requires re-administration of the viral
vector. However, following re-administration,
neutralizing antibodies are raised against both the fiber
25   and hexon proteins of the viral vector (Wohlfart, J.
Virology, 62, 2321-2328 (1988); Wohlfart et al., J.
Virology, 56, 896-903 (1985)). This antibody response
against the virus can prevent effective re-administration
of the viral vector.
30   Another drawback of using recombinant adenovirus in
gene therapy is that certain cells are not readily
amenable to adenovirus-mediated gene delivery. For
instance, lymphocytes, which lack the α_v integrin
adenoviral receptors, are impaired in the uptake of
35   adenoviruses (Silver et al., Virology 165, 377-387
(1988); Horvath et al., J. Virology, 62(1), 341-345
(1988)). This lack of ability to infect all cells has

lead researchers to seek out ways to introduce adenovirus into cells that cannot be infected by adenovirus, e.g. due to lack of adenoviral receptors. In particular, the virus can be coupled to a DNA-polylysine complex

5   containing a ligand (e.g., transferrin) for mammalian cells (e.g., Wagner et al., Proc. Natl. Acad. Sci., 89, 6099-6103 (1992); PCT patent application WO 95/26412). Similarly, adenoviral fiber protein can be sterically blocked with antibodies, and tissue-specific antibodies

10   can be chemically linked to the viral particle (Cotten et al., Proc. Natl. Acad. Sci. USA, 89, 6094-6098 (1992)).
         However, these approaches are disadvantageous in that they require additional steps that covalently link large molecules, such as polylysine, receptor ligands,

15   and antibodies, to the virus (Cotten (1992), supra; Wagner et al., Proc. Natl. Acad. Sci., 89, 6099-6103 (1992)). This adds to the size of the resultant vector as well as its cost of production. Moreover, the targeted particle complexes are not homogeneous in

20   structure, and their efficiency is sensitive to the relative ratios of viral particles, linking molecules, and targeting molecules used. Thus, this approach for expanding the repertoire of cells amenable to adenoviral-mediated gene therapy is less than optimal.

25   Recently, the efficiency of adenovirus-mediated gene transfer in vivo to even those cells which adenovirus has been reputed to enter with high efficiency has been called into question (Grubb et al., Nature, 371, 802-806 (1994); Dupuit et al., Human Gene Therapy, 6,

30   1185-1193 (1995)). The fiber receptor by means of which adenovirus initially contacts cells has not been identified, and its representation in different tissues has not been examined. It is generally assumed that epithelial cells in the lung and gut produce sufficient

35   levels of the fiber receptor to allow their optimal transduction. However, no studies have confirmed this point to date. In fact, studies have suggested that

adenovirus gene delivery to differentiated lung epithelium is less efficient than delivery to proliferating or to undifferentiated cells (Grubb et al., supra; Dupuit et al., supra).

Similarly, adenovirus has been shown to transduce a large number of tissues including lung epithelial cells (Rosenfeld et al., Cell, 68, 143-155 (1992)), muscle cells (Quantin et al., Proc. Natl. Acad. Sci., 89, 2581-2584 (1992)), endothelial cells (Lemarchand et al, Proc. Natl. Acad. Sci., 89, 6482-6486 (1992), fibroblasts (Anton et al., J. Virol., 69, 4600-4606 (1995), and neuronal cells (LaSalle et al., Science, 259, 988-990 (1993)). However, in many of these studies, very high levels of virus particles have been used to achieve transduction, often exceeding 100 plaque forming units (pfu)/cell, and corresponding to a multiplicity of infection (MOI) of 100. The requirement for a high MOI to achieve transduction is disadvantageous inasmuch as any immune response associated with adenoviral infection necessarily would be exacerbated with use of high doses.

Accordingly, there remains a need for vectors, such as adenoviral vectors, that are capable of infecting cells with a high efficiency, especially at lower MOIs, and that demonstrate an increased host cell range of infectivity. The present invention seeks to overcome at least some of the aforesaid problems of recombinant adenoviral gene therapy. In particular, it is an object of the present invention to provide a vector (such as an adenoviral vector) having a broad host range, and an ability to enter cells with a high efficiency, even at a reduced MOI, thereby reducing the amount of recombinant adenoviral vector administered and any side-effects/complications resulting from such administration. A further object of the present invention is to provide a method of gene therapy involving the use of a homogeneous adenovirus, wherein the viral particle is modified at the level of the adenoviral genome, without the need for

additional chemical modifications of viral particles.
These and other objects and advantages of the present
invention, as well as additional inventive features, will
be apparent from the following detailed description.

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BRIEF SUMMARY OF THE INVENTION

The present invention provides a chimeric adenoviral
coat protein (e.g., a fiber, hexon or penton protein),
which differs from the wild-type (i.e., native) fiber
10   protein by the introduction of a nonnative amino acid
sequence, preferably at or near the carboxyl terminus.
The resultant chimeric adenovirus coat protein is able to
direct entry into cells of a vector comprising the coat
protein that is more efficient than entry into cells of a
15   vector that is identical except for comprising a wild-
type adenovirus coat protein rather than the chimeric
adenovirus coat protein. One direct result of this
increased efficiency of entry is that the chimeric
adenovirus coat protein enables the adenovirus to bind to
20   and enter numerous cell types which adenovirus comprising
wild-type coat protein typically cannot enter or can
enter with only a low efficiency. The present invention
also provides an adenoviral vector that comprises the
chimeric adenovirus coat protein, and methods of
25   constructing and using such a vector.

BRIEF DESCRIPTION OF THE FIGURES
Figure 1 is a bar graph depicting the binding
(percent of input) of wild-type adenovirus to cells
30   derived from different tissues.
Figures 2A-B depict attachment of a nucleic acid
sequence at the end of the wild-type adenoviral fiber
gene (Fig. 2A) to derive a chimeric adenoviral fiber
protein (Fig. 2B) comprising a nonnative amino acid
35   sequence at the carboxy terminus. As indicated, the
length of the polyA tail, and, consequently, the number
of lysines in the resultant protein, can vary.

Figure 3 is a schematic diagram depicting the construction of the adenovirus transfer vector containing chimeric fiber protein pAd BS 59-100 UTV by way of intermediary transfer vectors. In particular, pAd NS 83-100 (also known as p193NS 83-100 or pNS 83-100) is used to derive fiber minus (i.e., F⁻) pAd NS 83-100 (also known as p193NS (ΔF) or pNS (ΔF)) (path A), pAd NS 83 100 (F⁻) is used to derive pAd NS 83-100 UTV (also known as p193NS (F5*), p193 (F5*), or pNS (F5*)) (path B), and pAd NS 83-100 UTV is used to derive pAd BS 59-100 UTV (path C).

Figures 4A-D depict the oligonucleotides employed for construction of GV10 UTV, i.e., the primers SEQ ID NO:9 (Fig. 4A), SEQ ID NO:10 (Fig. 4B), SEQ ID NO:11 (Fig. 4C), and SEQ ID NO:12 (Fig. 4D).

Figure 5 depicts a Western blot showing the size increase of the chimeric adenoviral fiber protein (UTV) as compared with the wild-type fiber protein (WT).

Figures 6A-B are graphs depicting a comparison of the binding of an adenoviral vector comprising wild-type fiber protein (i.e., GV10, open triangle) and adenoviral vector comprising chimeric fiber protein (i.e., GV10 UTV, filled circle) to a receptor-plus (A549, Fig. 6A) and a receptor-minus (HS 68, Fig. 6B) cell.

Figure 7 is a graph of UTV bound (counts per minute (CPM)) versus amount of competitor (µg/ml) for inhibition of binding of chimeric adenoviral fiber protein to receptor-minus cells (i.e., HS 68 fibroblasts) by the soluble factors chondroitin sulfate (open circle); heparin (filled circle); mucin (filled triangle); and salmon sperm DNA (open triangle).

Figure 8 is a graph of UTV bound (CPM) versus enzyme dilution for inhibition of binding of chimeric adenoviral fiber protein to receptor-minus cells (i.e., HS 68 fibroblasts) by the enzymes chondroitinase (open circles, stippled lines); heparinase (open circles, solid lines); and sialidase (triangles, solid lines).

Figure 9 is a bar graph depicting a comparison of
transfer of a *lacZ* reporter gene by an adenoviral vector
comprising wild-type fiber protein (i.e., GV10) and an
adenoviral vector comprising chimeric fiber protein
5   (i.e., GV10 UTV) as assessed by resultant reporter gene
expression (i.e., relative light units (RLU)) in various
receptor-plus and receptor-minus cells.

Figure 10 is a bar graph depicting a comparison of
transfer of a *lacZ* reporter gene by an adenoviral vector
10 comprising wild-type fiber protein (i.e., GV10) and an
adenoviral vector comprising chimeric fiber protein
(i.e., GV10 UTV) as assessed by resultant reporter
expression (i.e., relative light units (RLU)) in mouse
lung.

15   Figure 11 is a bar graph depicting the transfer of a
reporter gene (i.e., contained in pGUS) by an adenoviral
vector comprising wild-type fiber protein (i.e., GV10,
solid bars) and an adenoviral vector comprising chimeric
fiber (i.e., GV10 UTV, open bars) potentially bound via a
20 protein/DNA interaction into 293 cells, A549 cells, and
H700 T cells.

Figure 12 is a diagram that further depicts the
plasmid p193(F5*) (described as pAd NS 83-100 UTV in
Figure 3, and also known as p193 (F5*) or pNS (F5*)) used
25 to construct adenovirus fiber chimeras, and the sequence
of the C-terminus of the mutated fiber protein present in
the plasmid (polyadenylation site emboldened).

Figure 13 is a diagram that depicts plasmid p193NS
(F5*) pGS(K7) (also known as p193 (F5*) pGS(K7) or pNS
30 (F5*) pK7) used to construct adenovirus fiber chimeras.

Figure 14 is a diagram that depicts plasmid pBSS 75-
100 pGS(null) (also known as pBSS 75-100 ΔE3 pGS(null)).

Figure 15 is a diagram that depicts plasmid pBSS 75-
100 pGS(RK32) (also known as pBSS 75-100 ΔE3 pGS(RKKK)$_2$
35 or pBSS 75-100 ΔE3 pGS(RKKK2)).

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Figure 16 is a diagram that depicts plasmid pBSS 75-100 pGS(RK33) (also known as pBSS 75-100 ΔE3 pGS(RKKK), or pBSS 75-100 ΔE3 pGS(RKKK3)).

Figure 17 is a diagram that depicts plasmid p193NS F5F2K (also known as p193 F5F2K).

Figure 18 is a diagram that depicts plasmid p193NS F5F2K(RKKK)₂ (also known as p193NS F5F2K(RKKK2), p193NS F5F2K(RK32), or p193 F5F2K(RKKK2)).

Figure 19 is a diagram that depicts plasmid p193NS F5F2K(RKKK)₃ (also known as p193NS F5F2K(RKKK3), p193 F5F2K(RKKK3), or p193 F5FK(RK33)).

Figure 20 is a diagram that depicts plasmid pACT (RKKK)₃ (also known as pACT (RKKK3) or pACT (RK33)).

Figure 21 is a diagram that depicts plasmid pACT (RKKK)₂ (also known as pACT (RKKK2) or pACT (RK32)).

Figure 22 is a diagram that depicts plasmid pACT H11.

Figure 23 is a diagram that depicts plasmid pACT H11(RKKK)₂ (also known as pACT H11(RKKK2) or pACT H11(RK32)).

Figure 24 is a diagram that depicts plasmid p193 F5F9sK (also known as p193 F5F9K-Short).

Figure 25 is a diagram that depicts plasmid pSPdelta.

Figure 26 is a diagram that depicts plasmid pSP2alpha. The "j" indicates destroyed Ppu10I sites in the plasmid.

Figure 27 is a diagram that depicts plasmid pSP2alpha2. The "j" indicates destroyed Ppu10I sites in the plasmid.

Figure 28 is a graph of days post-infection versus FFU/cell for 293 cells infected with Ad5 (open circles). AdZ.F(RGD) (closed squares), or AdZ.F(pK7) (open triangles).

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DETAILED DESCRIPTION OF THE INVENTION

The present invention provides, among other things,
a recombinant adenovirus comprising a chimeric coat
protein, such as a chimeric fiber, penton, and/or hexon
protein. The chimeric coat protein comprises a nonnative
amino acid sequence, in addition to, or in place of, a
native amino acid sequence. This nonnative amino acid
sequence allows the chimeric fiber (or a vector
comprising the chimeric fiber) to more efficiently bind
to and enter cells.

Thus, the present invention provides, a chimeric
adenovirus coat protein comprising a nonnative amino acid
sequence, wherein the coat protein is able to direct
entry into cells of a vector comprising the coat protein
that is more efficient than entry into cells of a vector
that is identical except for comprising a wild-type
adenovirus coat protein rather than the chimeric
adenovirus coat protein (i.e., in the absence of the
chimeric adenovirus coat protein and in the presence of
the wild-type adenovirus coat protein).

Chimeric Coat Protein

A "coat protein" according to the invention
preferably comprises a fiber protein (especially an
adenoviral fiber protein), a penton protein (especially
an adenoviral penton protein), and a hexon protein
(especially an adenoviral hexon protein). In particular,
a coat protein preferably comprises an adenoviral fiber,
penton, or hexon protein. Any one of the serotypes of
human or nonhuman adenovirus can be used as the source of
the coat protein gene, optimally, however, the adenovirus
is an Ad2 or Ad5 adenovirus.

The coat protein is "chimeric" in that it comprises
amino acid residues that are not typically found in the
protein as isolated from wild-type adenovirus (i.e.,
comprising the native protein, or wild-type protein).
The coat protein thus comprises a "nonnative amino acid

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sequence". By "nonnative amino acid sequence" is meant
any amino acid sequence that is not found in the native
fiber of a given serotype of adenovirus and which
preferably is introduced into the fiber protein at the
5   level of gene expression (i.e., by introduction of a
"nucleic acid sequence that encodes a nonnative amino
acid sequence").

    Such a nonnative amino acid sequence comprises an
amino acid sequence (i.e., has component residues in a
10 particular order) which imparts upon the resultant
chimeric protein an ability to bind to and enter cells by
means of a novel cell surface binding site (i.e., a "UTV
sequence", or Universal Transfer Vector sequence), and/or
comprises a sequence incorporated to produce or maintain
15 a certain configuration of the resultant chimeric protein
(i.e., a "spacer sequence") between native/nonnative,
nonnative/nonnative, or a native/native sequence.
Inasmuch as the nonnative amino acid sequence is inserted
into or in place of an amino acid sequence, and the
20 manipulation of the amino acid sequence of the chimeric
coat protein preferably is made at the nucleic acid
level, the amino acid sequence that differs in the
chimeric coat protein from the wild-type coat protein
(i.e., the UTV sequence and the spacer sequence)
25 preferably can comprise an entirely nonnative amino acid
sequence, or a mixture of native and nonnative amino
acids).

    A "cell surface binding site" encompasses a receptor
(which preferably is a protein, carbohydrate,
30 glycoprotein, or proteoglycan) as well as any oppositely
charged molecule (i.e., oppositely charged with respect
to the chimeric coat protein, which preferably comprises
a nonnative amino acid sequence that is positively
charged, as described further herein) or other type of
35 molecule with which the chimeric coat protein can
interact to bind the cell, and thereby promote cell
entry. Examples of potential cell surface binding sites

include, but are not limited to: negatively charged heparin, heparan sulfate, hyaluronic acid, dermatan sulfate, and chondroitin sulfate moieties, for instance, found on glycosaminoglycans, and which further may

5   contain sialic acid, sulfate, and/or phosphate; sialic acid moieties found on mucins, glycoproteins, and gangliosides; major histocompatibility complex I (MHC I) glycoproteins; common carbohydrate components found in membrane glycoproteins, including mannose, N-acetyl-

10   galactosamine, N-acetyl-glucosamine, fucose, galactose, and the like; and phosphate moieties, for instance, on nucleic acids. However, a chimeric coat protein according to the invention, and methods of use thereof, is not limited to any particular mechanism of cellular

15   interaction (i.e., interaction with a particular cell surface binding site) and is not to be so construed.

Furthermore, such a cell surface binding site is "novel" inasmuch as the site is one that previously was inaccessible to interaction with an adenoviral coat

20   protein (i.e., wild-type adenoviral coat protein such as fiber protein), or was accessible only at a very low level, as reflected by the reduced efficiency of entry of a wild-type adenoviral coat protein-containing vector as compared with a vector comprising a chimeric adenovirus

25   coat protein such as fiber protein according to the invention. Moreover, the binding site is novel in that it is present on the majority of, if not all, cells, regardless of their origin. This is in contrast to the cellular binding site with which wild-type adenoviral

30   fiber protein is presumed to interact, which ostensibly is present only on a subset of cells, or is only accessible on a subset of cells, as reflected by the reduced efficiency of entry of a wild-type adenoviral fiber-containing vector.

35   "Efficiency of entry" can be quantitated by several means. In particular, efficiency of entry can be quantitated by introducing a chimeric coat protein into a

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vector, preferably a viral vector, and monitoring cell
entry (e.g., by vector-mediated delivery to a cell of a
gene such as a reporter gene) as a function of
multiplicity of infection (MOI)). In this case, a
5   reduced MOI required for cell entry of a vector
comprising a chimeric adenoviral coat protein as compared
with a vector that is identical except for comprising a
wild-type adenoviral coat protein rather than said
chimeric adenovirus coat protein, indicates "more
10   efficient" entry.

Similarly, efficiency of entry can be quantitated in
terms of the ability of vectors containing chimeric or
wild-type coat proteins, or the soluble chimeric or wild-
type coat proteins themselves, to bind to cells. In this
15   case, increased binding exhibited for the vector
containing a chimeric adenoviral coat protein, or the
chimeric coat protein itself, as compared with the
identical vector containing a wild-type coat protein
instead, or the wild-type coat protein itself, is
20   indicative of an increased efficiency of entry, or "more
efficient" entry.

A nonnative amino acid sequence according to the
invention preferably is inserted into or in place of an
internal coat protein sequence. Alternately, preferably
25   a nonnative amino acid sequence according to the
invention is at or near the C-terminus of a protein. In
particular, when a coat protein according to the
invention is a fiber protein, desirably a nonnative amino
acid sequence is at or near the C-terminus of the
30   protein. When a coat protein according to the invention
is a penton or hexon protein, preferably a nonnative
amino acid sequence is within an exposed loop of the
protein, e.g., as described in the following Examples,
particularly within a hypervariable region in loop 1
35   and/or loop 2 of the adenovirus hexon protein (Crawford-
Miksza et al., _J. Virol._, _70_, 1836-1844 (1996)). Thus,
desirably a nonnative amino acid sequence is in a region

of a coat protein that is capable of interacting with and binding a cell.

Furthermore, the method of the invention can be employed to create adenoviral vectors that contain UTV or UTV-like sequences in an extended (or spiked) structure, particularly in hexon and/or penton base protein, so as to result in lengthened hexon and/or penton base proteins, wherein the amino acid insertion projects outward from the protein as it is present in a virion capsid. In particular, such spiked coat proteins can be incorporated into a recombinant adenovirus along with a "short-shafted fiber" (further described herein), wherein the shaft of the fiber has been shortened, and, optionally, the knob of the fiber protein has been replaced with a knob (including the trimerization domain) of another serotype adenoviral vector from which the remainder of the fiber protein derived.

Accordingly, the short-shafted fiber protein preferably can be incorporated into an adenovirus having a chimeric penton base protein that comprises a UTV or UTV-like sequence, or having a "spiked" chimeric penton base protein that furthermore optionally can incorporate a UTV or UTV-like sequence. Also, the short-shafted fiber protein can be incorporated into an adenovirus having a chimeric hexon protein that comprises a UTV or UTV-like sequence, or having a "spiked" chimeric hexon protein that furthermore optionally can incorporate a UTV or UTV-like sequence.

Optimally, the nonnative amino acid sequence is linked to the protein by another nonnative amino acid sequence, i.e., by an intervening spacer sequence. A spacer sequence is a sequence that intervenes between the native protein sequence and the nonnative sequence, between a nonnative sequence and another nonnative sequence, or between a native sequence and another native sequence. A spacer sequence preferably is incorporated into the protein to ensure that the nonnative sequence

comprising the cell surface binding site projects from
the three dimensional structure of the chimeric protein
(especially the three dimensional structure of the
chimeric protein as it exists in nature, i.e., as part of

5   a capsid) in such a fashion so as to be able to interact
with and bind to cells. When a spacer sequence is
inserted into or replaces an internal coat protein
sequence, one or more spacer sequences may be present in
the chimeric coat protein.

10   An intervening spacer sequence can be of any suitable
length, preferably from about 3 to about 400 amino acids
for a spacer sequence added to derive a "spiked" coat
protein (as further described in the following Examples),
and preferably from about 3 to about 30 amino acids for

15   any other application described herein. A spacer
sequence can comprise any amino acids. Optimally, the
spacer sequence does not interfere with the functioning
of the coat protein in general, and the functioning of
the other nonnative amino acid sequence (i.e., the UTV or

20   UTV-like sequence) in particular.

         The nonnative amino acid sequence which is not a
spacer sequence, i.e., the UTV sequence, also can be of
any suitable length, preferably from about 3 to about 30
amino acids (although, optionally, as for the spacer

25   sequence, the UTV sequence can be longer, e.g., up to
about 400 amino acids). These amino acids preferably are
any positively charged residues that are capable of
binding to negatively charged moieties present on the
surface of a eukaryotic cell, and optimally are capable

30   of binding to negatively charged moieties that are
present on the surface of the majority of (if not all)
eukaryotic cells. In particular, such a negatively
charged moiety present on the surface of a eukaryotic
cell to which the UTV sequence binds includes the

35   aforementioned "cell surface binding site".

         Desirably the nonnative amino acid sequence
comprises amino acids selected from the group consisting

of lysine, arginine and histidine. Alternately, these
amino acids can be negatively charged residues that are
capable of binding to positively charged cell surface
binding sites, e.g., desirably the nonnative amino acid
sequence comprises amino acids selected from the group
consisting of aspartate and glutamate.

Thus, the nonnative amino acid sequence of a coat
protein preferably comprises a sequence selected from the
group consisting of SEQ ID NO:1 (i.e., Lys Lys Lys Lys
Lys Lys Lys Lys), SEQ ID NO:2 (i.e., Arg Arg Arg Arg Arg
Arg Arg Arg), and SEQ ID NO:3 (i.e., Xaa Xaa Xaa Xaa Xaa
Xaa Xaa Xaa, wherein "Xaa" comprises Lys or Arg), and
wherein 1, 2, 3, 4, or 5 residues of the sequence may be
deleted at the C-terminus thereof. When the coat protein
is a fiber protein, preferably the protein comprises a
sequence selected from the group consisting of SEQ ID
NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 (i.e., Gly
Ser Asn Lys Glu Ser Phe Val Leu Lys Lys Lys Lys Lys Lys),
and SEQ ID NO:5 (i.e., Ala Gly Ser Asn Lys Asn Lys Glu
Ser Phe Val Leu Lys Lys Lys Lys Lys Lys), and wherein 1,
2, 3, 4, or 5 residues of the sequence may be deleted at
the C-terminus thereof.

Also, sequences that bind that bind to heparin may
be involved in binding to a heparin-like receptor
(Sawitzky et al., Med. Microbiol. Immunol., 182, 285-92
(1993). Similarly, so-called "heparin binding sequences"
may mediate the interaction of the peptide or protein in
which they are contained with other cell surface binding
sites, such as with cell surface heparan sulfate
proteoglycan (Thompson et al., J. Biol. Chem., 269, 2541-
9 (1994)). Thus, preferably the nonnative amino acid
sequence (i.e., the UTV sequence) comprises these
sequences, as well as additional sequences that are
capable of recognizing a negatively charged moiety
broadly represented on the surface of eukaryotic cells.

In particular, preferably the nonnative amino acid
sequence comprises two basic amino acids (frequently Arg)

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located about 20 Å apart, facing in opposite directions
of an alpha helix (Margalit et al., J. Biol. Chem., 268,
19228-31 (1993); Ma et al., J. Lipid Res., 35, 2049-2059
(1994)). Other basic amino acids desirably are dispersed
5   between these two residues, facing one side, while
nonpolar residues face the other side, forming an
amphipathic structure, which optimally comprises the
motif XBBXBX [SEQ ID NO:49] or XBBBXXBX [SEQ ID NO:50],
where B is a basic amino acid (e.g., Lys, Arg, etc.), and
10   X is any other amino acid.

Also, preferably the UTV nonnative amino acid
sequence comprises: the sequence LIGRKKT [SEQ ID NO:51],
LIGRK [SEQ ID NO:52] or LIGRR [SEQ ID NO:53], which are
common heparin binding motifs present in fibronectin and
15   heat shock proteins (Hansen et al., Biochim. Biophys.
Acta, 1252, 135-45 (1995)); insertions of 7 residues of
either Lys or Arg, or mixtures of Lys and Arg (Fromm et
al., Arch. Biochem. Biophys., 323, 279-87 (1995)); the
common basic C-terminal region of IGFBP-3 and IGFBP-5 or
20   about 18 amino acids and which comprises a heparin
binding sequence (Booth et al., Growth Regul., 5, 1-17
(1995)); either one or both of the two hyaluronan (HA)
binding motifs located within a 35 amino acid region of
the C-terminus of the HA receptor RHAMM (Yang et al., J.
25   Cell Biochem., 56, 455-68 (1994)); a synthetic peptide
(Ala347-Arg361) modeled after the heparin-binding form of
Staphylococcus aureus vitronectin comprising heparin-
binding consensus sequences (Liang et al., J. Biochem.,
116, 457-63 (1994)); any one or more of five heparin
30   binding sites between amino acid 129 and 310 of bovine
herpesvirus 1 glycoprotein gIII or any one of four
heparin binding sites between amino acids 90 and 275 of
pseudorabies virus glycoprotein gIII (Liang et al.,
Virol., 194, 233-43 (1993)); amino acids 134 to 141 of
35   pseudorabies virus glycoprotein gIII (Sawitzky et al.,
Med. Microbiol. Immunol., 182, 285-92 (1993); heparin
binding regions corresponding to charged residues at

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positions 279-282 and 292-304 of human lipoprotein lipase
(Ma et al., supra); a synthetic 22 residue peptide, N22W,
with a sequence NVSPPRRARVTDATETTITISW [SEQ ID NO:54] or
residues TETTITIS [SEQ ID NO:55] of this synthetic

5   peptide modeled after fibronectin and which exhibit
heparin binding properties (Ingham et al., Arch. Biochem.
Biophys., 314, 242-246 (1994)); GVEFVCCP [SEQ ID NO:56]
motif present in the ectodomain zinc binding site of the
Alzheimer beta-amyloid precursor protein (APP), as well

10   as various other APP-like proteins, which modulates
heparin affinity (Bush et al., J. Biol. Chem., 229,
26618-21 (1994)); 8 amino acid residue peptides derived
from the cross-region of the laminin A chain (Tashiro et
al., Biochem. J., 302, 73-9 (1994)); synthetic peptides

15   based on the heparin binding regions of the serine
protease inhibitor antithrombin III including peptides
F123-G148 and K121-A134 (Tyler-Cross et al., Protein
Sci., 3, 620-7 (1994)); a 14 K N-terminal fragment of APP
and a region close to the N-terminus (i.e., residues 96

20   110) proposed as heparin binding regions (Small et al.,
J. Neurosci., 14, 2117-27 (1994)); a stretch of 21 amino
acids of the heparin binding epidermal growth factor-like
growth factor (HB-EGF) characterized by a high content of
lysine and arginine residues (Thompson et al., J. Biol.

25   Chem., 269, 2541-9 (1994)); a 17 amino acid region
comprising the heparin binding region of thrombospondin
and corresponding to a hep 1 synthetic peptide (Murphy-
Ullrich et al., J. Biol. Chem., 268, 26784-9 (1993)); a
23 amino acid sequence (Y565-A587) of human von

30   Willebrand factor that binds heparin (Tyler-Cross et
al., Arch. Biochem. Biophys., 306, 528-33 (1993)); the
fibronectin-derived peptide PRARI [SEQ ID NO:57] (and
larger peptides comprising this motif, such as WQPPRARI
[SEQ ID NO:58]) that binds heparin (Woods et al., Mol.

35   Biol. Cell., 4, 605-613 (1993); the heparin binding
region of platelet factor 4 (Sato et al., Jpn. J. Cancer
Res., 84, 485-8 (1993); and the K18K sequence in the

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fibroblast growth factor receptor tyrosine kinase
transmembrane glycoprotein (Kan et al., Science, 259,
1918-21 (1993)).

Moreover, the UTV sequence can comprise other
sequences that are described in the Examples which
follow. Thus, preferably the UTV sequence is selected
from the group consisting of [SEQ ID NO:1], [SEQ ID
NO:2], [SEQ ID NO:3], [SEQ ID NO:4], [SEQ ID NO:5], [SEQ
ID NO:20], [SEQ ID NO:22], [SEQ ID NO:24], [SEQ ID
NO:26], [SEQ ID NO:28], [SEQ ID NO:30], [SEQ ID
NO:32], [SEQ ID NO:34], [SEQ ID NO:36], [SEQ ID NO:38],
[SEQ ID NO:40], [SEQ ID NO:42], [SEQ ID NO:46], [SEQ ID
NO:48], [SEQ ID NO:49], [SEQ ID NO:50], [SEQ ID NO:51],
[SEQ ID NO:52], [SEQ ID NO:53], [SEQ ID NO:54], [SEQ ID
NO:55], [SEQ ID NO:56], [SEQ ID NO:57], [SEQ ID NO:58],
[SEQ ID NO:73], [SEQ ID NO:74], [SEQ ID NO:76], [SEQ ID
NO:78], [SEQ ID NO:90], and [SEQ ID NO:93]. These
sequences also can be employed wherein 1, 2, or 3
residues of the sequence are deleted at the C- or N-
termins. Also, inasmuch as a spacer sequence can be any
sequence of amino acids that does not interfere with the
functioning of the protein, according to the invention,
any of the aforementioned UTV sequences also can comprise
spacer sequences.

It also is preferable that the nonnative amino acid
sequence comprise amino acid sequences that are
"equivalents" of any of the aforementioned sequences
(i.e., are "UTV-like sequences"). An equivalent can be a
sequence that carries out the same function (with perhaps
minor differences in efffectiveness), and yet may differ
slightly in terms of its amino acid sequence, or other
structural features. In particular, an equivalent
sequence is one that comprises one or more conservative
amino acid substitutions of the sequence. A "conservative
amino acid substitution" is an amino acid substituted by
an alternative amino acid of similar charge density,
hydrophilicity/hydrophobicity, size, and/or configuration

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(e.g., Val for Ile). In comparison, a "nonconservative
amino acid substitution" is an amino acid substituted by
an alternative amino acid of differing charge density,
hydrophilicity/hydrophobicity, size, and/or configuration
5   (e.g., Val for Phe).

Nucleic Acid Encoding a Chimeric Coat Protein
        As indicated previously, preferably the nonnative
amino acid sequence is introduced at the level of DNA.
10   Accordingly, the invention preferably also provides an
isolated and purified nucleic acid encoding a coat
protein according to the invention, wherein the nucleic
acid sequence that encodes the nonnative amino acid
sequence comprises a sequence of SEQ ID NO:6 (i.e., GGA
15   TCC AA), which is located prior to the polyadenylation
site. Similarly, the invention preferably provides an
isolated and purified nucleic acid comprising a sequence
selected from the group consisting of SEQ ID NO:7 (i.e.,
GGA TCC AAT AAA GAA TCG TTT GTG TTA TGT) and SEQ ID NO:9
20   (i.e., GCC GGA TCC AAC AAG AAT AAA GAA TCG TTT GTG TTA),
[SEQ ID NO:19], [SEQ ID NO:21], [SEQ ID NO:23], [SEQ ID
NO:25], [SEQ ID NO:27], [SEQ ID NO:29], [SEQ ID NO:31],
[SEQ ID NO:33], [SEQ ID NO:35], [SEQ ID NO:37], [SEQ ID
NO:39], [SEQ ID NO:41], [SEQ ID NO:45], [SEQ ID NO:47],
25   [SEQ ID NO:72], [SEQ ID NO:75], [SEQ ID NO:77], and [SEQ
ID NO:89]. The invention further provides conservatively
modified variants of these nucleic acids.
        A "conservatively modified variant" is a variation
on the nucleic acid sequence that results in a
30   conservative amino acid substitution. In comparison, a
"nonconservatively modified variant" is a variation on
the nucleic acid sequence that results in a
nonconservative amino acid substitution. The means of
making such modifications are well known in the art, are
35   described in the Examples which follow, and also can be
accomplished by means of commercially available kits and
vectors (e.g., New England Biolabs, Inc., Beverly, MA;

Clontech, Palo Alto, CA). Moreover, the means of
assessing such substitutions (e.g., in terms of effect on
ability to bind and enter cells) are described in the
Examples herein.

5   The means of making such a chimeric coat protein,
particularly the means of introducing the sequence at the
level of DNA, is well known in the art, is illustrated in
Figure 2 for a representative chimeric protein, and is
described in the Examples that follow. Briefly, the

10   method comprises introducing a sequence (preferably the
sequence of SEQ ID NO:6 or a conservatively modified
variant thereof) into the sequence of the coat protein.
In one preferred embodiment described in the Examples
which follow, the introduction is made prior to any stop

15   codon or polyadenylation signal so as to induce a
frameshift mutation into the resultant protein, such that
the chimeric protein incorporates additional amino acids.

          Generally, this can be accomplished by cloning the
fiber sequence into a plasmid or some other vector for

20   ease of manipulation of the sequence. Then, restriction
sites flanking the sequence at which the frameshift
mutation is to be introduced are identified. A double-
stranded synthetic oligonucleotide is created from
overlapping synthetic single-stranded sense and antisense

25   oligonucleotides (e.g., from the sense and antisense
oligonucleotides, respectively, TAT GGA GGA TCC AAT AAA
GAA TCG TTT GTG TTA TGT TTC AAC GTG TTT ATT TTT C [SEQ ID
NO:9] and AAT TGA AAA ATA AAC ACG TTG AAA CAT AAC ACA AAC
GAT TCT TTA TTG GAT CCT CCA [SEQ ID NO:10], as

30   illustrated in Figure 4) such that the double-stranded
oligonucleotide incorporates the restriction sites
flanking the target sequence. The plasmid or other
vector is cleaved with the restriction enzymes, and the
oligonucleotide sequence having compatible cohesive ends

35   is ligated in to the plasmid or other vector, to replace
the wild-type DNA. Other means of in vitro site-directed
mutagenesis such as are known to those skilled in the

art, and can be accomplished (in particular, using PCR),
for instance, by means of commercially available kits,
can be used to introduce the mutated sequence into the
coat protein coding sequence.

5   Once the sequence is introduced into the chimeric
coat protein, the nucleic acid fragment encoding the
sequence can be isolated, e.g., by PCR amplification
using 5' and 3' primers. For instance, with respect to a
chimeric fiber protein, the fragment can be isolated by

10   PCR using the primer TCCC CCCGGG TCTAGA TTA GGA TCC TTC
TTG GGC AAT GTA TGA [SEQ ID NO:11], and the primer CGT
GTA TCC ATA TGA CAC AGA [SEQ ID NO:12], as illustrated in
Figure 4. Use of these primers in this fashion results
in an amplified chimeric fiber-containing fragment that

15   is flanked by restriction sites (i.e., in this case *Nde*I
and *Bam*HI sites) that can be used for convenient
subcloning of the fragment. Other means of generating a
chimeric coat protein also can be employed.

       Thus, the frameshift mutation can be introduced into

20   any part of a coat protein coding sequence. With respect
to SEQ ID NO:6, for instance, this sequence can be placed
at the region of the coat protein gene that codes for the
C-terminus of the protein (i.e., can be added immediately
prior to the TAA stop codon), or can be placed earlier

25   into the coding region, such as between codons coding for
Ala (i.e., A) and Gln (i.e., Q) to produce the
aforementioned coding sequence of SEQ ID NO:8, which
encodes a chimeric protein comprising the sequence of SEQ
ID NO:5. Similarly, this approach can be employed to

30   introduce a frameshift even earlier in the coding
sequence, e.g., either inserted into or in place of an
internal (i.e., native) coat protein sequence.

       Moreover, the double-stranded oligonucleotide can
also incorporate a further restriction site that also can

35   be employed in manipulating the sequence. For instance,
the sequence of SEQ ID NO:6 introduced in the vector
comprises a modified *Bam*HI site, i.e., the site is

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"modified" in that it adds additional nucleotides onto
the palindromic recognition sequence. This sequence also
can be synthesized to comprise any other restriction site
convenient for DNA manipulations. When incorporated into
5   the coat protein coding sequence, the sequence not only
introduces a frame shift mutation, but also can be used
to introduce other coding sequences into the coat protein
gene. In particular, the coding sequences introduced in
this fashion can comprise codons for lysine, arginine and
10   histidine, or codons for aspartate and glutamate, either
alone, or in any combination. Furthermore, a new
translation stop codon can follow these codons for the
amino acids, allowing a chimeric protein to be produced
that only incorporates a given number of additional amino
15   acids in the nonnative amino acid sequence. The codons
for the amino acids and the translation stop codon can be
introduced into the frameshift mutation-inducing novel
restriction site incorporated into the coat protein by
synthesizing oligonucleotides comprising these sequences
20   that are flanked by the restriction site as previously
described (e.g., that comprise 5' and 3' BamHI sites), or
by other such means that are known to those skilled in
the art.

The size of the DNA used to replace the native
25   receptor binding sequence may be constrained, for
example, by impeded folding of the fiber or improper
assembly of the penton base/fiber complex. DNA encoding
the aforementioned amino acid sequences (e.g., lysine,
arginine, histidine, aspartate, glutamate, and the like)
30   is preferred for insertion into the fiber gene sequence
in which the native receptor binding sequence has been
deleted or otherwise mutated. Moreover, other DNA
sequences, such as those that encode amino acids for
incorporation into spacer sequences, preferably are used
35   to replace the native coat protein coding sequence.

Vector Comprising a Chimeric Coat Protein

A "vector" according to the invention is a vehicle
for gene transfer as that term is understood by those
skilled in the art. Four types of vectors encompassed by
5   the invention are plasmids, phages, viruses, and
liposomes.   Plasmids, phages, and viruses can be
transferred to a cell in their nucleic acid form, and
liposomes can be employed to transfer nucleic acids.
Hence, the vectors that can be employed for gene transfer
10   are referred to herein as "transfer vectors".

Preferably, a vector according to the invention is a
virus, especially a virus selected from the group
consisting of nonenveloped viruses, i.e., nonenveloped
RNA or DNA viruses.   Also, a virus can be selected from
15   the group consisting of enveloped viruses, i.e.,
enveloped RNA or DNA viruses.   Such viruses preferably
comprise a coat protein.   Desirably, the viral coat
protein is one that projects outward from the capsid such
that it is able to interact with cells.   In the case of
20   enveloped RNA or DNA viruses, preferably the coat protein
is in fact a lipid envelope glycoprotein (i.e., a so-
called spike or peplomer).

In particular, preferably a vector is a nonenveloped
virus (i.e., either a RNA or DNA virus) from the family
25   *Hepadnaviridae*, *Parvoviridae*, *Papovaviridae*,
Adenoviridae, or *Picornaviridae*.   A preferred
nonenveloped virus according to the invention is a virus
of the family *Hepadnaviridae*, especially of the genus
Hepadnavirus.   A virus of the family *Parvoviridae*
30   desirably is of the genus *Parvovirus* (e.g., parvoviruses
of mammals and birds) or *Dependovirus* (e.g., adeno-
associated viruses (AAVs)).   A virus of the family
Papovaviridae preferably is of the subfamily
Papillomavirinae (e.g., the papillomaviruses including,
35   but not limited to, human papillomaviruses (HPV) 1-48) or
the subfamily *Polyomavirinae* (e.g., the polyomaviruses
including, but not limited to, JC, SV40 and BK virus).   A

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virus of the family *Adenoviridae* desirably is of the
genus *Mastadenovirus* (e.g., mammalian adenoviruses) or
Aviadenovirus (e.g., avian adenoviruses). A virus of the
family *Picornaviridae* is preferably a hepatitis A virus
5   (HAV), hepatitis B virus (HBV), or a non-A or non-B
hepatitis virus.

Similarly, a vector can be an enveloped virus from
the family *Herpesviridae* or *Retroviridae*, or can be a
Sindbis virus. A preferred enveloped virus according to
10   the invention is a virus of the family *Herpesviridae*,
especially of the subfamily or genus *Alphaherpesvirinae*
(e.g., the herpes simplex-like viruses), *Simplexvirus*
(e.g., herpes simplex-like viruses), *Varicellavirus*
(e.g., varicella and pseudorabies-like viruses),
15   *Betaherpesvirinae* (e.g., the cytomegaloviruses),
Cytomegalovirus (e.g., the human cytomegaloviruses),
Gammaherpesvirinae (e.g., the lymphocyte-associated
viruses), and *Lymphocryptovirus* (e.g., EB-like viruses).

Another preferred enveloped virus is a RNA virus of
20   the family *Retroviridae* (i.e., preferably is a
retrovirus), particularly a virus of the genus or
subfamily *Oncovirinae*, *Spumavirinae*, *Spumavirus*,
Lentivirinae, and *Lentivirus*. A RNA virus of the
subfamily *Oncovirinae* is desirably a human T-lymphotropic
25   virus type 1 or 2 (i.e., HTLV-1 or HTLV-2) or bovine
leukemia virus (BLV), an avian leukosis-sarcoma virus
(e.g., Rous sarcoma virus (RSV), avian myeloblastosis
virus (AMV), avian erythroblastosis virus (AEV), Rous-
associated virus (RAV)-1 to 50, RAV-0), a mammalian C-
30   type virus (e.g., Moloney murine leukemia virus (MuLV),
Harvey murine sarcoma virus (HaMSV), Abelson murine
leukemia virus (A-MuLV), AKR-MuLV, feline leukemia virus
(FeLV), simian sarcoma virus, reticuloendotheliosis virus
(REV), spleen necrosis virus (SNV)), a B-type virus
35   (e.g., mouse mammary tumor virus (MMTV)), or a D-type
virus (e.g., Mason-Pfizer monkey virus (MPMV), "SAIDS"
viruses). A RNA virus of the subfamily *Lentivirus* is

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desirably a human immunodeficiency virus type 1 or 2
(i.e., HIV-1 or HIV-2, wherein HIV-1 was formerly called
lymphadenopathy associated virus 3 (HTLV-III) and
acquired immune deficiency syndrome (AIDS)-related virus

5   (ARV)), or another virus related to HIV-1 or HIV-2 that
has been identified and associated with AIDS or AIDS-like
disease. The acronym "HIV" or terms "AIDS virus" or
"human immunodeficiency virus" are used herein to refer
to these HIV viruses, and HIV-related and -associated

10   viruses, generically. Moreover, a RNA virus of the
subfamily Lentivirus preferably is a Visna/maedi virus
(e.g., such as infect sheep), a feline immunodeficiency
virus (FIV), bovine lentivirus, simian immunodeficiency
virus (SIV), an equine infectious anemia virus (EIAV), or

15   a caprine arthritis-encephalitis virus (CAEV).
        An especially preferred vector according to the
invention is an adenoviral vector (i.e., a viral vector
of the family Adenoviridae, optimally of the genus
Mastadenovirus). Desirably such a vector is an Ad2 or

20   Ad5 vector, although other serotype adenoviral vectors
can be employed. The adenoviral vector employed for gene
transfer can be wild-type (i.e., replication competent).
Alternately, the adenoviral vector can comprise genetic
material with at least one modification therein, which

25   can render the virus replication deficient. The
modification to the adenoviral genome can include, but is
not limited to, addition of a DNA segment, rearrangement
of a DNA segment, deletion of a DNA segment, replacement
of a DNA segment, or introduction of a DNA lesion. A DNA

30   segment can be as small as one nucleotide and as large as
36 kilobase pairs (i.e., the approximate size of the
adenoviral genome) or, alternately, can equal the maximum
amount which can be packaged into an adenoviral virion
(i.e., about 38 kb). Preferred modifications to the

35   adenoviral genome include modifications in the E1, E2, E3
or E4 region. Similarly, an adenoviral vector can be a
cointegrate, i.e., a ligation of adenoviral sequences,

with other sequences, such as other virus or plasmid
sequences.

In terms of a viral vector (e.g., particularly a
replication deficient adenoviral vector), such a vector
5   can comprise either complete capsids (i.e., including a
viral genome such as an adenoviral genome) or empty
capsids (i.e., in which a viral genome is lacking, or is
degraded, e.g., by physical or chemical means). Along
the same lines, since methods are available for
10   transferring viruses, plasmids, and phages in the form of
their nucleic acid sequences (i.e., RNA or DNA), a vector
(i.e., a transfer vector) similarly can comprise RNA or
DNA, in the absence of any associated protein such as
capsid protein, and in the absence of any envelope lipid.
15   Similarly, since liposomes effect cell entry by fusing
with cell membranes, a transfer vector can comprise
liposomes (e.g., such as are commercially available, for
instance, from Life Technologies, Bethesda, MD), with
constitutive nucleic acids encoding the coat protein.
20   Thus, according to the invention whereas a vector
"comprises" a chimeric adenoviral coat protein, a
transfer vector "encodes" a chimeric adenoviral coat
protein; liposome transfer vectors in particular "encode"
in the sense that they contain nucleic acids which, in
25   fact, encode the protein.

A vector according to the invention can comprise
additional sequences and mutations, e.g., some within the
coat protein itself. For instance, a vector according to
the invention further preferably comprises a nucleic acid
30   comprising a passenger gene.

A "nucleic acid" is a polynucleotide (DNA or RNA).
A "gene" is any nucleic acid sequence coding for a
protein or a nascent RNA molecule. A "passenger gene" is
any gene which is not typically present in and is
35   subcloned into a vector (e.g., a transfer vector)
according to the present invention, and which upon
introduction into a host cell is accompanied by a

discernible change in the intracellular environment
(e.g., by an increased level of deoxyribonucleic acid
(DNA), ribonucleic acid (RNA), peptide or protein, or by
an altered rate of production or degradation thereof). A

5   "gene product" is either an as yet untranslated RNA
molecule transcribed from a given gene or coding sequence
(e.g., mRNA or antisense RNA) or the polypeptide chain
(i.e., protein or peptide) translated from the mRNA
molecule transcribed from the given gene or coding

10   sequence. Whereas a gene comprises coding sequences plus
any non-coding sequences, a "coding sequence" does not
include any non-coding (e.g., regulatory) DNA. A gene or
coding sequence is "recombinant" if the sequence of bases
along the molecule has been altered from the sequence in

15   which the gene or coding sequence is typically found in
nature, or if the sequence of bases is not typically
found in nature. According to this invention, a gene or
coding sequence can be wholly or partially synthetically
made, can comprise genomic or complementary DNA (cDNA)

20   sequences, and can be provided in the form of either DNA
or RNA.

Non-coding sequences or regulatory sequences include
promoter sequences. A "promoter" is a DNA sequence that
directs the binding of RNA polymerase and thereby

25   promotes RNA synthesis. "Enhancers" are cis-acting
elements of DNA that stimulate or inhibit transcription
of adjacent genes. An enhancer that inhibits
transcription is also termed a "silencer". Enhancers
differ from DNA-binding sites for sequence-specific DNA

30   binding proteins found only in the promoter (which are
also termed "promoter elements") in that enhancers can
function in either orientation, and over distances of up
to several kilobase pairs, even from a position
downstream of a transcribed region. According to the

35   invention, a coding sequence is "operably linked" to a
promoter (e.g., when both the coding sequence and the
promoter constitute a passenger gene) when the promoter

is capable of directing transcription of that coding
sequence.

Accordingly, a "passenger gene" can be any gene, and
desirably is either a therapeutic gene or a reporter
5   gene. Preferably a passenger gene is capable of being
expressed in a cell in which the vector has been
internalized. For instance, the passenger gene can
comprise a reporter gene, or a nucleic acid sequence
which encodes a protein that can in some fashion be
10   detected in a cell. The passenger gene also can comprise
a therapeutic gene, for instance, a therapeutic gene
which exerts its effect at the level of RNA or protein.
For instance, a protein encoded by a transferred
therapeutic gene can be employed in the treatment of an
15   inherited disease, such as, e.g., the cystic fibrosis
transmembrane conductance regulator cDNA for the
treatment of cystic fibrosis. The protein encoded by the
therapeutic gene may exert its therapeutic effect by
resulting in cell killing. For instance, expression of
20   the gene in itself may lead to cell killing, as with
expression of the diphtheria toxin A gene, or the
expression of the gene may render cells selectively
sensitive to the killing action of certain drugs, e.g.,
expression of the HSV thymidine kinase gene renders cells
25   sensitive to antiviral compounds including acyclovir,
gancyclovir and FIAU (1-(2-deoxy-2-fluoro-β-D-
arabinofuranosil)-5-iodouracil).

Moreover, the therapeutic gene can exert its effect
at the level of RNA, for instance, by encoding an
30   antisense message or ribozyme, a protein which affects
splicing or 3' processing (e.g., polyadenylation), or can
encode a protein which acts by affecting the level of
expression of another gene within the cell (i.e., where
gene expression is broadly considered to include all
35   steps from initiation of transcription through production
of a processed protein), perhaps, among other things, by
mediating an altered rate of mRNA accumulation, an

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alteration of mRNA transport, and/or a change in post-
transcriptional regulation. Accordingly, the use of the
term "therapeutic gene" is intended to encompass these
and any other embodiments of that which is more commonly

5   referred to as gene therapy and is known to those of
skill in the art. Similarly, the recombinant adenovirus
can be used for gene therapy or to study the effects of
expression of the gene in a given cell or tissue *in vitro*
or *in vivo*.

10   The recombinant adenovirus comprising a chimeric
coat protein such as a fiber protein and the recombinant
adenovirus that additionally comprises a passenger gene
or genes capable of being expressed in a particular cell
can be generated by use of a transfer vector, preferably

15   a viral or plasmid transfer vector, in accordance with
the present invention. Such a transfer vector preferably
comprises a chimeric adenoviral coat protein gene
sequence as previously described. The chimeric coat
protein gene sequence comprises a nonnative sequence in

20   place of the native sequence, which has been deleted, or
in addition to the native sequence.

        A recombinant chimeric coat protein gene sequence
(such as a fiber gene sequence) can be moved from an
adenoviral transfer vector into baculovirus or a suitable

25   prokaryotic or eukaryotic expression vector for
expression and evaluation of receptor or protein
specificity and avidity, trimerization potential, penton
base binding, and other biochemical characteristics.

        Accordingly, the present invention also provides

30   recombinant baculoviral and prokaryotic and eukaryotic
expression vectors comprising a chimeric adenoviral coat
protein gene sequence (preferably a fiber gene sequence),
which also are "transfer vectors" as defined herein. The
chimeric coat protein gene sequence (e.g., fiber gene

35   sequence) includes a nonnative sequence in addition to or
in place of a native amino acid sequence, and which
enables the resultant chimeric coat protein (e.g., fiber

protein) to bind to a binding site other than a binding
site bound by the native sequence. By moving the
chimeric gene from an adenoviral vector to baculovirus or
a prokaryotic or eukaryotic expression vector, high
protein expression is achievable (approximately 5-50% of
the total protein being the chimeric fiber).

A vector according to the invention further can
comprise, either within, in place of, or outside of the
coding sequence of a coat protein additional sequences
that impact upon the ability of a coat protein such as
fiber protein to trimerize, or comprise a protease
recognition sequence. A sequence that impacts upon the
ability to trimerize is one or more sequences that enable
trimerization of a chimeric coat protein that is a fiber
protein. A sequence that comprises a protease
recognition sequence is a sequence that can be cleaved by
a protease, thereby effecting removal of the chimeric
coat protein (or a portion thereof) and attachment of the
recombinant adenovirus to a cell by means of another coat
protein. When employed with a coat protein that is a
fiber protein, the protease recognition site preferably
does not affect fiber trimerization or receptor
specificity of the fiber protein. For instance, in one
embodiment of the present invention, preferably the fiber
protein, or a portion thereof, is deleted in by means of
a protease recognition sequence, and then the novel cell
surface binding site is incorporated into either the
penton base or hexon coat protein, preferably with use of
a spacer sequence as previously described.

In terms of the production of vectors and transfer
vectors according to the invention, transfer vectors are
constructed using standard molecular and genetic
techniques such as are known to those skilled in the art.
Vectors (e.g., virions or virus particles) are produced
using viral vectors. For instance, a viral vector
comprising a chimeric coat protein according to the
invention can be constructed by providing to a cell that

does not comprise any E4 complementing sequences: (1) a linear vector comprising the chimeric fiber and the wild-type E4 gene, and (2) a linear vector that is E4⁻, as illustrated in **Figure 3**. As described in the Examples

5 which follow, this methodology results in recombination between the sequences, generating a vector that comprises a portion of the initial E4⁻ vector and a portion of the E4⁺ vector, particularly the region comprising the chimeric fiber sequences.

10 Similarly, the fiber chimera-containing particles are produced in standard cell lines, e.g., those currently used for adenoviral vectors. Following production and purification, the particles in which fiber is to be deleted are rendered fiberless through digestion

15 of the particles with a sequence-specific protease, which cleaves the fiber proteins and releases them from the viral particles to generate fiberless particles. For example, thrombin recognizes and cleaves at known amino acid sequences that can be incorporated into the vector

20 (Stenflo et al., _J. Biol. Chem._, _257_, 12280-12290 (1982)). Similarly, deletion mutants lacking the fiber gene can be employed in vector construction, e.g., H2_dl_802, H2_dl_807, and H2_dl_1021 (Falgout et al., _J. Virol._, _62_, 622-625 (1988). These fiberless particles

25 have been shown to be stable and capable of binding and infecting cells (Falgout et al., _supra_). These resultant particles then can be targeted to specific tissues via the penton base or other coat protein, preferably such other coat protein that comprises one or more nonnative

30 amino acid sequences according to the invention.

Alternately, recombinant adenovirus comprising chimeric fiber protein having further modifications can be produced by the removal of the native knob region, which comprises receptor-binding and trimerization

35 domains, of the fiber protein and its replacement with a nonnative trimerization domain (Peteranderl et al., _Biochemistry_, _31_, 12272-12276 (1992)) and a nonnative

amino acid sequence according to the invention. A
recombinant adenovirus comprising a chimeric fiber
protein also can be produced by point mutation in the
knob region and the isolation of clones that are capable

5   of trimerization. In either case, and also with respect
to the removal and replacement of the native receptor-
specific binding sequence as described above, new protein
binding domains can be added onto the C-terminus of the
fiber protein or into exposed loops of the fiber protein

10   by inserting one or more copies of the nucleic acid
sequence encoding the nonnative amino acid sequence into
the appropriate position. Preferably, such a fiber
protein is able to trimerize, so that it is able to bind
to penton base protein.

15   The method described above for generating chimeric
fiber protein also can be used to make other chimeric
coat proteins, e.g., chimeric hexon or penton protein.

Illustrative Uses

20   The present invention provides a chimeric protein
that is able to bind to cells and mediate entry into
cells with high efficiency, as well as vectors and
transfer vectors comprising same. The chimeric coat
protein itself has multiple uses, e.g., as a tool for

25   studies *in vitro* of adenovirus binding to cells (e.g., by
Scatchard analysis as shown previously by Wickham et al.
(1993), supra), to block binding of adenovirus to
receptors *in vitro* (e.g., by using antibodies, peptides,
and enzymes, as described in the Examples), and to

30   protect against adenoviral infection *in vivo* by competing
for binding to the binding site by which adenovirus
effects cell entry.

          A vector comprising a chimeric coat protein also can
be used in strain generation and as a means of making new

35   vectors. For instance, the nonnative amino acid sequence
can bind to nucleic acids, and can be introduced
intracellularly as a means of generating new vectors via

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recombination. Similarly, a vector can be used in gene therapy. For instance, a vector of the present invention can be used to treat any one of a number of diseases by delivering to targeted cells corrective DNA, i.e., DNA

5   encoding a function that is either absent or impaired, or a discrete killing agent, e.g., DNA encoding a cytotoxin that, for example, is active only intracellularly. Diseases that are candidates for such treatment include, for example, cancer, e.g., melanoma, glioma or lung

10   cancers; genetic disorders, e.g., cystic fibrosis, hemophilia or muscular dystrophy; pathogenic infections, e.g., human immunodeficiency virus, tuberculosis or hepatitis; heart disease, e.g., preventing restenosis following angioplasty or promoting angiogenesis to

15   reperfuse necrotic tissue; and autoimmune disorders, e.g., Crohn's disease, colitis or rheumatoid arthritis.

     In particular, gene therapy can be carried out in the treatment of diseases, disorders, or conditions associated with different tissues that ostensibly lack

20   high levels of the receptor to which wild-type adenovirus fiber protein binds, and thus for which current adenoviral-mediated approaches to gene therapy are less than optimal (e.g., for delivery to monocyte/macrophages, fibroblasts, neuronal, smooth muscle, and epithelial

25   cells). Tissues comprised of these cells (and diseases, disorders, or conditions associated therewith) include, but are not limited to: endothelia (e.g., angiogenesis, restenosis, inflammation, and tumors); neuronal tissue (e.g., tumors and Alzheimer's disease); epithelium (e.g.,

30   disorders of the skin, cornea, intestine, and lung); hematopoietic cells (e.g., human immunodeficiency virus (HIV-1, HIV-2), cancers, and anemias); smooth muscle (e.g., restenosis); and fibroblasts (e.g., inflammation).

     Moreover, instead of transferring a therapeutic gene,

35   a reporter gene, or some type of marker gene can be transferred instead using the vectors (particularly the adenoviral vectors) of the invention. Marker genes and

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reporter genes are of use, for instance, in cell differentiation and cell fate studies, as well as potentially for diagnostic purposes. Moreover, a standard reporter gene such as a β-galactosidase reporter gene, a
5   gene encoding green fluorescent protein (GFP), or a β-glucuronidase gene can be used *in vivo*, e.g., as a means of assay in a living host, or, for instance, as a means of targeted cell ablation (see, e.g., Minden et al., Biotechniques, 20, 122-129 (1996); Youvan, Science, 268,
10 264 (1995); U.S. Patent 5,432,081; Deonarain et al., Br. J. Cancer, 70, 786-794 (1994)).

      Similarly, it may be desirable to transfer a gene to use a host essentially as a means of production *in vivo* of a particular protein. Along these lines, transgenic
15 animals have been employed, for instance for the production of recombinant polypeptides in the milk of transgenic bovine species (e.g., PCT International Application WO 93/25567). Other "non-therapeutic" reasons for gene transfer include the study of human diseases using an
20 animal model (e.g., use of transgenic mice and other transgenic animals including p53 tumor suppressor gene knockouts for tumorigenic studies, use of a transgenic model for impaired glucose tolerance and human Alzheimer's amyloid precursor protein models for the study of glucose
25 metabolism and pathogenesis of Alzheimer's disease, respectively, etc.)

      These aforementioned illustrative uses are by no means comprehensive, and it is intended that the present invention encompasses such further uses which flow from,
30 but are not explicitly recited, in the disclosure herein. Similarly, there are numerous advantages associated with the use of the various aspects of the present invention.

      For instance, use of a universal targeting vector according to the invention is advantageous inasmuch as:
35 (1) the vector can potentially be used for all cells and tissues; (2) only one vector is required for use in all cell lines, there is no need for co-transfecting an

independent vector; (3) the vector is capable of
effecting gene delivery with an efficiency that is
increased over that observed for vectors comprising wild-
type fiber protein; (4) the vector, unlike prior vectors,
5   does not target specific cells, but instead increases
transduction efficiency in what appears to be a global
fashion; (5) the vector is capable of mediating gene
transfer when employed at a reduced dose (i.e.,
multiplicity of infection (MOI)) as compared with vector
10 comprising wild-type fiber protein, and thus likely
reduces the dosage-related drawbacks that accompany
currently available adenoviral vectors; and (6) the
vector can be propagated and maintained using currently
available cell lines.

15   The ability of a universal targeting vector such as
a universal targeting adenovirus vector to potentially
bind to and enter all or most tissues has several
advantages. These advantages include increased gene
delivery efficiency to multiple tissues, the availability
20 of a single vector capable of delivering genes to all
tissues, and simplified production of necessary
components for gene delivery. Moreover, such a universal
targeting vector comprises a potential to deliver
exogenous DNA into cells by "piggy backing" the DNA on
25 the vector by means of a protein/DNA interaction.

       Further potential advantages of such a universal
targeting vector include a substantially increased
efficiency of delivery (e.g., increased by 10- to 100-
fold) into cells expressing low levels of fiber receptor
30 to which wild-type fiber protein binds, as well as
increased efficiency into cells or tissues expressing
fiber receptor to which wild-type fiber binds. Moreover
the reduced dosage at which the vectors are employed
should result in a decrease in adenovirus-associated
35 inflammation, the humoral response to adenovirus, and the
cytotoxic T-lymphocyte response to adenovirus.

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Furthermore, the vector is advantageous in that it
can be isolated and purified by conventional means.
Since changes in the vector are made at the genome level,
there are no cumbersome and costly post-production
5   modifications required, as are associated with other
vectors (see, e.g., Cotten et al., _supra_; Wagner et al.,
supra). Similarly, special receptor-expressing cells
lines are not required. A UTV vector can be propagated
to similar titers as a wild-type vector lacking the fiber
10   modification.

Means of Administration

The vectors and transfer vectors of the present
invention can be employed to contact cells either _in_
15   _vitro_ or _in vivo_. According to the invention
"contacting" comprises any means by which a vector is
introduced intracellularly; the method is not dependent
on any particular means of introduction and is not to be
so construed. Means of introduction are well known to
20   those skilled in the art, and also are exemplified
herein.

Accordingly, introduction can be effected, for
instance, either _in vitro_ (e.g., in an _ex vivo_ type
method of gene therapy or in tissue culture studies) or
25   _in vivo_ by electroporation, transformation, transduction,
conjugation or triparental mating, (co-)transfection,
(co-)infection, membrane fusion with cationic lipids,
high velocity bombardment with DNA-coated
microprojectiles, incubation with calcium phosphate-DNA
30   precipitate, direct microinjection into single cells, and
the like. Similarly, the vectors can be introduced by
means of cationic lipids, e.g., liposomes. Such
liposomes are commercially available (e.g., Lipofectin®,
Lipofectamine™, and the like, supplied by Life
35   Technologies, Gibco BRL, Gaithersburg, MD). Moreover,
liposomes having increased transfer capacity and/or
reduced toxicity _in vivo_ (see, e.g., PCT patent

application WO 95/21259) can be employed in the present invention. Other methods also are available and are known to those skilled in the art.

According to the invention, a "host" (and thus a "cell" from a host) encompasses any host into which a vector of the invention can be introduced, and thus encompasses an animal, including, but not limited to, an amphibian, bird, fish, insect, reptile, or mammal. Optimally a host is a mammal, for instance, rodent, primate (such as chimpanzee, monkey, ape, gorilla, orangutan, or gibbon), feline, canine, ungulate (such as ruminant or swine), as well as, in particular, human.

Inasmuch as a universal targeting vector ostensibly enters all cells, a cell can be any cell into which such a vector can enter. In particular, a universal targeting vector can be employed for gene transfer to a cell that expresses low or undetectable levels of fiber receptor, including, but not limited to, an endothelial, smooth muscle, neuronal, hematopoietic, or fibroblast cell.

One skilled in the art will appreciate that suitable methods of administering a vector (particularly an adenoviral vector) of the present invention to an animal for purposes of gene therapy (see, for example, Rosenfeld et al., _Science_, _252_, 431-434 (1991); Jaffe et al., _Clin._ _Res._, _39(2)_, 302A (1991); Rosenfeld et al., _Clin. Res._, _39(2)_, 311A (1991); Berkner, _BioTechniques_, _6_, 616-629 (1988)), chemotherapy, and vaccination are available, and, although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. Pharmaceutically acceptable excipients also are well-known to those who are skilled in the art, and are readily available. The choice of excipient will be determined in part by the particular method used to administer the recombinant vector. Accordingly, there is a wide variety of suitable formulations for use in the context of the present invention. The following methods

and excipients are merely exemplary and are in no way
limiting.

Formulations suitable for oral administration can
consist of (a) liquid solutions, such as an effective
amount of the compound dissolved in diluents, such as
water, saline, or orange juice; (b) capsules, sachets or
tablets, each containing a predetermined amount of the
active ingredient, as solids or granules; (c) suspensions
in an appropriate liquid; and (d) suitable emulsions.
Tablet forms can include one or more of lactose,
mannitol, corn starch, potato starch, microcrystalline
cellulose, acacia, gelatin, colloidal silicon dioxide,
croscarmellose sodium, talc, magnesium stearate, stearic
acid, and other excipients, colorants, diluents,
buffering agents, moistening agents, preservatives,
flavoring agents, and pharmacologically compatible
excipients. Lozenge forms can comprise the active
ingredient in a flavor, usually sucrose and acacia or
tragacanth, as well as pastilles comprising the active
ingredient in an inert base, such as gelatin and
glycerin, or sucrose and acacia, emulsions, gels, and the
like containing, in addition to the active ingredient,
such excipients as are known in the art.

A vector or transfer vector of the present
invention, alone or in combination with other suitable
components, can be made into aerosol formulations to be
administered via inhalation. These aerosol formulations
can be placed into pressurized acceptable propellants,
such as dichlorodifluoromethane, propane, nitrogen, and
the like. They may also be formulated as pharmaceuticals
for non-pressured preparations such as in a nebulizer or
an atomizer.

Formulations suitable for parenteral administration
include aqueous and non-aqueous, isotonic sterile
injection solutions, which can contain anti-oxidants,
buffers, bacteriostats, and solutes that render the
formulation isotonic with the blood of the intended

recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

Additionally, a vector or transfer vector of the present invention can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases.

Formulations suitable for vaginal administration can be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

The dose administered to an animal, particularly a human, in the context of the present invention will vary with the gene of interest, the composition employed, the method of administration, and the particular site and organism being treated. However, the dose should be sufficient to effect a therapeutic response.

As previously indicated, a vector or a transfer vector of the present invention also has utility *in vitro*. Such a vector can be used as a research tool in the study of adenoviral attachment and infection of cells and in a method of assaying binding site-ligand interaction. Similarly, the recombinant coat protein comprising a nonnative amino acid sequence in addition to or in place of a native receptor binding sequence can be used in receptor-ligand assays and as adhesion proteins *in vitro* or *in vivo*, for example.

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The following examples further illustrate the present invention and, of course, should not be construed as in any way limiting its scope.

5   Example 1

This example describes an investigation of the levels of adenovirus receptor in different cells, as determined by the ability of wild-type adenovirus to bind to the cells.

10   For these experiments, the ability of adenovirus comprising wild-type fiber to bind to cells derived from various tissues was assessed. Adenovirus particles of an Ad5 strain were labeled with [^{3}H]-thymidine as previously described (see, e.g., Wickham et al., Cell, 73, 309-319

15   (1993)). Subsaturating levels of thymidine-labeled adenovirus were added to 200 µl of 10^6 cells preincubated about 30 to 60 minutes with or without 20 µg/ml of soluble fiber protein. The cells were incubated with the virus for 1 hour at 4°C and then washed 3 times with cold

20   phosphate buffered saline (PBS). The remaining cell-associated counts were measured in a scintillation counter. Specific binding was measured by subtracting the cell-associated counts (i.e., counts per minute (cpm)) in the presence of fiber from the cell-associated

25   counts in the absence of fiber. Binding in the presence of fiber was never more than 2% of the total input of radioactive virus particles. Results were obtained as the average of triplicate measurements.

As illustrated in **Figure 1**, a substantial number of

30   the cells derived from different tissues expressed little or no fiber receptor, as indicated by a relative inability of wild-type adenovirus to bound to these cells. Cells of epithelial origin (i.e., "receptor-plus" cells including Chang, HeLa, and A549 cells) bound high

35   levels of adenovirus. In comparison, non-epithelial cells (i.e., "receptor-minus" cells such as monocyte/macrophages, fibroblasts, neuronal, smooth

muscle, and epithelial cells) exhibited about 10-fold or
more reductions in virus binding as compared to
epithelial-like cells.

5   These results confirm the previously unrecognized
relative inability of adenovirus to bind to and hence
enter receptor-minus non-epithelial cells, as compared
with receptor-plus epithelial cells. Presumably this
inability is due to the low representation of receptors
for wild-type adenoviral fiber protein on these cells.
10

Example 2

This example describes the construction of an
adenoviral vector comprising a chimeric coat protein,
particularly a chimeric adenoviral fiber protein.
15   To overcome the transduction limitation imposed by
the presence of only a limited number of fiber receptors
on clinically relevant tissues such as non-epithelial
tissue, a modified adenovirus vector was constructed as
depicted in Figures 2A and 2B to derive a vector that is
20   referred to herein as a "universal transfer vector", or
UTV. In particular, a frameshift mutation was created in
a gene encoding an adenoviral coat protein, in this case,
in the fiber gene. In wild-type adenovirus, the
unmodified fiber gene contains a nested translational
25   stop signal (TAA) and transcriptional polyadenylation
signal (AATAAA). The polyadenylation signal directs the
addition of a polyA tail onto the 3' end of the
transcript. The polyA tail typically comprises anywhere
from about 20 to about 200 nucleotides. Following
30   transcription and exit from the nucleus, the TAA stop
signal directs termination of translation by the
ribosome.

In comparison, the modified fiber gene of a UTV
vector lacks an in-frame translational "stop" signal.
35   Following normal transcription and addition of the polyA
extension onto the mRNA, in the absence of the stop
codon, the ribosome continues translation of the

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transcript into the polyA region. Inasmuch as the codon
AAA codes for the amino acid lysine, the resultant
chimeric fiber gene translation product produced by a UTV
contains an addition of a string of polylysine residues
at the C-terminus, i.e., Lys Lys Lys Lys Lys Lys Lys Lys
[SEQ ID NO:1]. It is possible that a cellular process
acts to limit the length of the polylysine string, since
the polylysine residues typically comprise from about 3
to about 30 residues in the chimeric fiber protein.
Whatever the case, however, the polylysine protein
modification, as well as further modifications described
herein, allows the UTV to efficiently attach to cells
lacking high levels of the receptor for wild-type
adenoviral fiber protein (i.e., receptor-minus cells).

In terms of vector construction and
characterization, standard molecular and genetic
techniques, such as the generation of strains, plasmids,
and viruses, gel electrophoresis, DNA manipulations
including plasmid isolation, DNA cloning and sequencing,
Western blot assays, and the like, were performed such as
are known to those skilled in the art, and as are
described in detail in standard laboratory manuals (e.g.,
Maniatis et al., Molecular Cloning: A Laboratory Manual,
2nd ed. (Cold Spring Harbor, NY, 1992); Ausubel et al.,
Current Protocols in Molecular Biology, (1987)).
Restriction enzymes and other enzymes used for molecular
manipulations were purchased from commercial sources
(e.g., Boehringer Mannheim, Inc., Indianapolis, Indiana;
New England Biolabs, Beverly, Massachusetts; Bethesda
Research Laboratories, Bethesda, Maryland), and were used
according to the recommendations of the manufacturer.
Cells employed for experiments (e.g., cells of the
transformed human embryonic kidney cell line 293 (i.e.,
CRL 1573 cells) and other cells supplied by American Type
Culture Collection) were cultured and maintained using
standard sterile culture reagents, media and techniques,

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as previously described (Erzerum et al., <u>Nucleic Acids Research</u>, <u>21</u>, 1607-1612 (1993)).

Accordingly, the frameshift mutation of the fiber stop codon was created by introducing a modified *BamHI* site (i.e., GGATCCAA [SEQ ID NO:6]) into an adenoviral transfer vector. This was done as illustrated in Figure 3 by starting with the transfer plasmid pAd NS 83-100 (which also is known as p193NS 83-100 or pNS 83-100). pAd NS 83-100 was constructed by cloning the Ad5 *NdeI* to *SalI* fragment, which spans the 83-100 map unit region of the Ad5 genome containing the fiber gene, into the plasmid pNEB193 (New England Biolabs, Beverly, MA).

The *NdeI-MunI* fragment of pAd NS 83-100 was replaced with a synthetic oligonucleotide comprising a *BamHI* site, which was flanked by a 5' *NdeI* site and a 3' *MunI* site to facilitate cloning. The double-stranded synthetic oligonucleotide fragment was created from overlapping synthetic single-stranded sense and antisense oligonucleotides, i.e., respectively, the sense primer TAT GGA GGA TCC AAT AAA GAA TCG TTT GTG TTA TGT TTC AAC GTG TTT ATT TTT C [SEQ ID NO:9], and the antisense primer AAT TGA AAA ATA AAC ACG TTG AAA CAT AAC ACA AAC GAT TCT TTA TTG GAT CCT CCA [SEQ ID NO:10], as illustrated in **Figures 4A** and **4B**, respectively. The ends of the overlapping oligomers were made to have overhangs compatible for direct cloning into the *NdeI* and *MunI* sites.

The resultant transfer plasmid, pAd NS 83-100 (F⁻) (which also is known as p193NS (ΔF) or pNS (ΔF)), lacks all but the first 50 base pairs of the coding sequence for the fiber gene (i.e., is "fiber-minus"). The vector furthermore contains the entire adenovirus E4 coding sequence. The plasmid retains the AATAAA polyadenylation signal included in the synthetic *NdeI/MunI* oligonucleotide, and also incorporates the new *BamHI* restriction site.

The mutated fiber gene was incorporated into the fiber-minus pAd NS 83-100 plasmid using synthetic sense and antisense oligonucleotide primers to amplify the fiber gene with use of the polymerase chain reaction

5   (PCR) while incorporating a modified *Bam*HI site following the last codon of the fiber gene to create the mutant fiber gene. This incorporated modified *Bam*HI site also serves to code for the amino acids glycine and serine, resulting in a chimeric nucleic acid sequence of GGA TCC

10 AAT AAA GAA TCG TTT GTG TTA TGT [SEQ ID NO:7]. The modified fiber gene thus codes for an extension to the resultant chimeric fiber protein of Gly Ser Asn Lys Glu Ser Phe Val Leu Lys Lys Lys [SEQ ID NO:4], wherein the length of the polylysine string can vary. The synthetic

15 oligonucleotides employed for fiber amplification were the primer TCCC CCCGGG TCTAGA TTA GGA TCC TTC TTG GGC AAT GTA TGA [SEQ ID NO:11], and the primer CGT GTA TCC ATA TGA CAC AGA [SEQ ID NO:12], as illustrated in **Figures 4C** and **4D**, respectively.

20   The amplified gene product was then cut with the restriction enzymes *Nde*I and *Bam*HI, and was cloned into the *Nde*I/*Bam*HI sites of the fiber-minus plasmid pAd NS 83-100 to create the transfer vector pAd NS 83-100 UTV (which also is known as p193NS (F5*), p193 (F5*), or pNS

25 (F5*)). The entire *Nde*I to *Sal*I adenovirus sequence of pAd NS 83-100 UTV was cloned into the fiber-minus plasmid pAd BS 59-100 to create pAd BS 59-100 UTV (which also is known as p193NS (F5*), p193 (F5*), or pNS (F5*).

       The UTV adenovirus vector was created through

30 homologous recombination in 293 cells. Namely, the E4· pAd BS 59-100 UTV transfer vector was linearized with *Sal*I, and was transfected into 293 cells that were previously infected with the adenovirus vector, A2F. The A2F vector was derived from a GV10 vector. The Ad5-based

35 vector GV10 contains the *lacZ* gene under the control of the Rous sarcoma virus promoter (i.e., comprises RSV *lacZ*). The insertion of the reporter gene in GV10 is

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made within the E1 region (i.e., the vector is E1⁻). The
GV10 vector also contains a deletion of the E3 region,
but is E4⁺. In comparison with GV10 (i.e., RSV lacZ E1⁻
E3⁻ E4⁺), A2F further comprises a deletion of the
5   essential E4 adenovirus genes, but is E3⁺ (i.e., RSV lacZ
E1⁻ E3⁺ E4⁻).

The 293 cells contain an E1 complementing sequence,
but do not contain an E4 complementing sequence. The
lack of an E4 complementing sequence prevents replication
10   of the E4⁻ A2F vector in the 293 cell line. However, upon
co-introduction of A2F virus and pAd BS 59-100 UTV in 293
cells, homologous recombination takes place between the
UTV transfer vector and the A2F adenoviral genome,
producing an E3⁺ E4⁺ adenovirus genome comprising a
15   chimeric fiber protein, which is capable of replication
in 293 cells. This particular resultant UTV vector was
designated GV10 UTV.

The GV10 UTV vector was isolated using standard
plaque isolation techniques with 293 cells. Following
20   three successive rounds of plaque-purification, the GV10
UTV vector contained the fiber mutation and was free of
any contamination by the E4⁻ A2F vector. The presence of
the chimeric fiber sequences in the GV10 UTV vector was
confirmed by sequencing the fiber mRNA using reverse
25   transcriptase-polymerase chain reaction (RT-PCR), which
validated the presence of a polyadenine tail in the
chimeric fiber mRNA.

Similarly, the production of a chimeric fiber
protein by the vector was confirmed by Western blot. To
30   accomplish this, 293 cells were infected at a
multiplicity of infection (MOI) of 5 with either GV10
comprising wild-type adenoviral fiber protein or with
GV10 UTV comprising chimeric fiber protein. At two days
post-infection, the cells were washed and then lysed in
35   PBS by three freeze-thaw cycles. The lysates were
cleared by centrifugation and loaded onto a 10% sodium
dodecyl sulfate/polyacrylamide gel. Following

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electrophoresis, the proteins were transferred onto
nitrocellulose and detected by chemiluminescence using a
polyclonal antibody to fiber. The Western blot is
depicted in **Figure** 5. As can be seen from this figure,
5   the migration of the proteins indicates that the chimeric
UTV fiber is about 1.5 to about 2.0 kilodaltons larger
than the unmodified 62 kilodalton WT fiber protein.

        These results confirm that the method identified
herein can be employed to introduce modifications into
10   the fiber protein to produce a chimeric fiber protein.
Similar techniques can be employed to introduce
modifications into the hexon or penton proteins, or to
introduce similar modifications (e.g., the addition of a
string of amino acids comprised of arginine, lysine
15   and/or histidine, or comprised of aspartate and/or
glutamate, or the addition of any of these sequences into
a coding region of the coat proteins).

                              Example 3
20   This example describes the binding to cells of an
adenoviral vector comprising a chimeric coat protein such
as a chimeric fiber protein as compared with a wild-type
adenoviral vector, either in the presence or absence of
added soluble wild-type fiber protein
25   For these experiments, the cells identified in
Example 1 to which adenovirus binds with either high
efficiency (i.e. receptor-plus cells) or low efficiency
(i.e., receptor-minus cells) were employed. The
epithelial cell line A549 was used as representative of
30   receptor-plus cells, and the fibroblast cell line HS 68
was used as representative of receptor-minus cells.
Confluent monolayers of either A549 or HS 68 cells were
preincubated at 4°C with concentrations of soluble fiber
protein ranging from 0 to about 10 µg/ml. The GV10 UTV
35   vector comprising chimeric fiber protein (UTV) or GV10
vector comprising wild-type fiber protein (WT) were
labeled with tritiated thymidine as described in Example

1.   About 20,000 cpm of [³H]-thymidine labeled GV10 UTV or
GV10 vector were then incubated with the cells for about
2 hours at 4°C.   The cells were washed three times with
cold PBS, and the cell-associated cpm were determined by
5   scintillation counting.   Results were obtained as the
average of duplicate measurements and are presented in
Figures 6A and 6B for the A549 and HS 68 cell lines,
respectively.

As can be seen in **Figures 6A-B**, the GV10 UTV vector
10   chimeric fiber protein was able to bind both receptor-
plus (**Fig. 6A**) and receptor-minus (**Fig. 6B**) cells with
high efficiency.   In comparison, the GV10 vector
comprising wild-type fiber was more effective at binding
to receptor-plus cells.   In particular, radiolabeled GV10
15   UTV bound to cells expressing detectable levels of fiber
receptor (i.e., A549 alveolar epithelial cells) about 2-
to 2.5-fold better than GV10.   Whereas all of the binding
of the GV10 vector was inhibited by competing recombinant
fiber protein, only about 40% of the GV10 UTV vector was
20   inhibited by the addition of competing fiber.   No
detectable binding of GV10 vector comprising wild-type
adenoviral fiber to HS 68 human foreskin fibroblast cells
lacking fiber receptor was observed.   In comparison, the
GV10 UTV vector efficiently bound to HS 68 cells, and the
25   addition of competing fiber protein had no effect on
binding.

These results confirm that binding of the GV10 UTV
vector comprising a chimeric coat protein (i.e., a
chimeric fiber protein) does not occur via the wild-type
30   adenoviral fiber receptor, and instead occurs via a
heretofore unrecognized fiber receptor.   Moreover, the
results confirm that incorporation of a chimeric coat
protein such as a chimeric fiber protein into an
adenoviral vector results in an improved adenoviral
35   vector..   Namely, the modification comprised by the GV10
UTV vector enables it overcome the aforementioned
relative inability of wild-type adenovirus to bind to

50

receptor-minus cells, in particular, non-epithelial
cells, and also allows the modified vector to bind to
receptor-plus cells with an increased efficiency.

5   Example 4
        This example describes an investigation of the
ability of various soluble factors, and inhibitors of
these soluble factors, to block binding of adenovirus
comprising chimeric fiber protein to receptor-minus HS 68
10   fibroblast cells.
        For these experiments, the inhibition of GV10 UTV
binding by various negatively charged molecules including
salmon sperm DNA, mucin, chondroitin sulfate, and
heparin, was assessed. Chondroitin sulfate and heparin
15   are negatively charged molecules which get their charge
from sulfate groups. Mucin is negatively charged due to
the presence of sialic acid moieties, and DNA is
negatively charged due to its incorporation of phosphate
moieties. About 20,000 cpm of UTV in 250 µl of binding
20   buffer (i.e., Dulbecco's Modified Eagle Media (D-MEM) was
incubated at room temperature for about 30 minutes with
concentrations of negatively charged molecules ranging
from about 1 x 10^{-3} to about 1 x 10^4 µg/ml. Following
incubation, the mixtures were chilled on ice, and were
25   then added to prechilled HS 68 cells plated in 24 well
plates. The cells were incubated for about 1 hour, and
then the cells were washed three times with PBS. Cell-
associated cpm were determined by scintillation counting,
and reported as the average of duplicate measurements.
30   As indicated in **Figure 7**, whereas the presence of
competing wild-type fiber protein had no effect on
binding of a GV10 UTV vector (i.e., comprising chimeric
fiber) to HS 68 cells, negatively-charged competing
molecules were able to block GV10 UTV binding.   All four
35   molecules were able to inhibit GV10 UTV binding to HS 68
cells, although heparin and DNA were most effective.
These molecules have no significant effect on the binding

51

of a GV10 vector (i.e., comprising wild-type fiber) to
cells expressing high levels of fiber receptor (i.e.,
A549 cells; data not shown).

These results confirm that negatively charged

5   molecules are able to block binding of the GV10 UTV
vector to cells mediated by chimeric fiber protein. This
inhibition presumably is due to the binding of the
negatively charged molecules to the positively charged
polylysine residues present on the GV10 UTV fiber.

10   Accordingly, the impact of enzymes which cleave these
negatively charged molecules on binding to cells of the
GV10 UTV vector was assessed.

HS 68 cells were plated in 24 well plates, and were
preincubated with the dilutions of heparinase (Sigma, St.

15   Louis, MO), chondroitinase (Sigma), and sialidase
(Boehringer Mannheim, Inc.) ranging from about 0.0001 to
1 for 45 minutes at 37°C, followed by 15 minutes at 4°C.
Whereas chondroitinase cleaves chondroitin sulfate,
heparinase cleaves heparin and heparin sulfate, and

20   sialidase cleaves sialic acid. The initial starting
concentrations for dilutions were as follows: heparinase,
25 U/ml (U = 0.1 μmole/hour, pH = 7.5, 25°C);
chondroitinase, 2.5 U/ml (U = 1.0 μmole/minute, pH = 8.0,
37°C); and sialidase 0.25 U/ml (U = 1.0 μmole/minute, pH =

25   5.5, 37°C). Following incubation, the cells were washed
three times with cold PBS, and were then incubated with
20,000 cpm of labeled GV10 UTV vector for about 1 hour at
4°C. The cells were then washed three times with cold
PBS, and the cell-associated cpm were determined by

30   scintillation counting. The results were reported as the
average of duplicate measurements.

As illustrated in **Figure 8**, pretreatment of HS 68
cells with enzymes that remove negatively charged
molecules from the cell surface confirms that the GV10

35   UTV vector comprising the chimeric fiber protein
interacts with negatively charged sites on the cell
surface. In particular, heparinase and sialidase were

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both able to reduce GV10 UTV binding, although heparinase
was more effective than sialidase on HS 68 cells.

    Thus, these results confirm that a vector comprising
chimeric fiber protein (e.g. a GV10 UTV vector), unlike

5   wild-type adenovirus, interacts in a novel fashion with
negatively charged molecules on the cell surface to
effect cell entry. These results further demonstrate
that a vector comprising negatively charged residues
(e.g., aspartate and glutamate) instead of positively

10   charged molecules (e.g., lysine) similarly can be
employed to bind to and effect cell entry via positively
charged molecules present on the cell surface.

<u>Example 5</u>

15   This example evaluate gene delivery to different
types of cells mediated by an adenoviral vector
comprising chimeric coat protein such as chimeric fiber
protein (e.g., GV10 UTV) as compared to gene delivery
mediated by adenovirus comprising wild-type coat protein

20   such as fiber protein (e.g., GV10).

    For these experiments, the relative levels of *lacZ*
gene delivery by a vector containing the wild-type fiber
protein (i.e., GV10) as compared with vector containing
chimeric fiber protein (i.e., GV10 UTV) were compared in

25   epithelial-like cells (i.e., HeLa, A549, HepG2 and H700 T
cells), smooth muscle cells (i.e., HA SMC and HI SMC
cells), endothelial cells (i.e., HUVEC and CPAE cells),
fibroblast cells (i.e., HS 68 and MRC-5 cells),
glioblastoma cells (i.e., U118 cells) and monocyte

30   macrophages (i.e., THP-1 cells). Approximately 2×10^5
cells were inoculated one day prior to transduction by
adenovirus into 24 multiwell plates. Each well was then
infected at an MOI of 1 with GV10 (i.e., comprising wild-
type adenoviral fiber protein) or with GV10 UTV (i.e.,

35   comprising chimeric adenoviral fiber) in a 250 μl volume
for about one hour. The wells were then washed and
incubated for two days, after which the *lacZ* activity of

53

the cell lysates was determined. The results were
reported as the average of duplicate measurements.

As illustrated in **Figure 9**, the use of the GV10 UTV
vector to transfer a reporter gene to a panel of cell
5   lines confirms that the presence of the chimeric fiber
protein (UTV) increases *lacZ* gene delivery to cells
expressing low or undetectable levels of fiber receptor
(i.e., receptor-minus cells) from about 5- to about 300-
fold as compared with wild-type vector (GV10). In cells
10   expressing high levels of the fiber receptor (i.e.,
receptor-plus cells), the incorporation of the chimeric
fiber protein in the GV10 UTV vector results in an
increase in gene delivery of up to about 3-fold.

This reduction in expression observed with
15   transduction of receptor-minus non-epithelial cells as
compared with receptor-plus epithelial cells by
adenovirus comprising a wild-type fiber protein (i.e.,
GV10) directly correlates with the relative ability of
the vector to bind these different cell types, as
20   reported in Example 3. These results support the view
that the low expression of receptors for wild-type
adenovirus fiber protein is a significant limiting factor
to their efficient transduction by current adenovirus
vectors.

25   Similarly, the ability of the chimeric coat protein
(i.e., the chimeric fiber protein) to augment gene
transfer *in vivo* was assessed. Three BALB/c mice were
inoculated intranasally with about 1×10^8 pfu of GV10 in
50 µl of a saline solution comprising 10 mM $MgCl_2$ and 10
30   mM Tris (pH 7.8). Another three mice received the same
dose of GV10 UTV, and two mice received the saline
solution alone. The animals were sacrificed at two days
post-administration, and the lungs were assayed for *lacZ*
activity. The lungs were prepared for analysis by snap-
35   freezing the lung in liquid nitrogen, grinding the tissue
with a mortar and pestle, and lysing the ground tissue in
1.0 ml of *lacZ* reporter lysis buffer (Promega Corp.,

54

Madison, WI). A fluorometric assay was used to monitor
lacZ activity, and the results of the experiments were
reported as the average activity measured from each group
of animals.

5   The results of these experiments are illustrated in
Figure 10. As can be seen from this Figure, gene
transfer *in vivo* mediated by the GV10 UTV vector
comprising chimeric fiber protein (UTV) as compared with
a vector comprising wild-type fiber protein (GV10)

10   resulted in an average of 8-fold higher delivery to mouse
lung.

These results thus confirm that incorporation of a
chimeric coat protein (in this case, a chimeric fiber
protein) in an adenoviral vector substantially increases

15   the efficiency of vector-mediated gene delivery both *in
vitro* and *in vivo* as compared to an adenovirus vector
comprising wild-type fiber protein. Moreover, the
results support the conclusion that low fiber receptor
expression is a significant factor contributing to the

20   suboptimal delivery observed in the lung and in other
tissues. Also, the results confirm the superiority of
the GV10 UTV vector, as well as other similar UTV
vectors, over other currently available adenoviral
vectors for gene transfer (e.g., delivery of the CFTR

25   gene) to the lung and other tissues.

Example 6

This example evaluates the ability of a vector
according to the invention comprising a chimeric coat

30   protein (e.g., a chimeric fiber protein) to interact with
passenger DNA by means of a protein/DNA interaction, and
to thereby carry the DNA into the cell in a "piggy-back"
fashion.

For these experiments, an adenoviral vector

35   comprising wild-type fiber (i.e., GV10) and an adenoviral
vector comprising chimeric fiber (i.e., GV10 UTV) were
used to assess gene transfer to receptor-plus epithelial

cells (i.e., 293, A549, and H700 T cells). In control
experiments, the cells were transduced with the vectors
as previously described. In the experimental condition,
the vectors were incubated with the plasmid pGUS, which

5   comprises a β-glucuronidase reporter gene, such that the
chimeric adenoviral fiber protein was able to complex
with the plasmid DNA. Specifically, about 5 x 10⁷ active
particles (i.e., fluorescence focus units (ffu)) of GV10
or GV10 UTV were incubated for 1 hour with about 2.5 µg

10   of plasmid pGUS DNA. The mixture was then added to about
2 x 10⁵ of the indicated cells in 250 µl of DMEM
containing 10% fetal bovine serum. Both β-glucuronidase
and β-galactosidase activity were then assessed by
fluorometric assay at 10 days post-transduction. β-

15   glucuronidase expression in cells was monitored similarly
to the β-galactosidase assay for *lacZ* expression, by
monitoring the generation of a blue color when β-
glucuronidase catalyzes a reaction with the substrate X-
glu.

20   The results of these experiments are illustrated in
Figure 11. Comparable levels of *lacZ* expression were
obtained when either a GV10 vector (i.e. comprising wild-
type fiber protein) or a GV10 UTV vector (i.e. comprising
chimeric fiber protein) were employed to transfer the

25   reporter gene in *cis* to epithelial cells. In comparison,
the wild-type vector was able to transfer intracellularly
the plasmid pGUS at only a relatively low level in all
epithelial cells, as assessed by β-glucuronidase gene
expression. This basal level of gene transfer likely was

30   accomplished by means of receptor-mediated uptake (RME)
of bystander molecules, as previously described (PCT
patent application WO 95/21259). However, with use of a
GV10 UTV vector comprising a chimeric fiber protein,
transfer of the pGUS plasmid was substantially increased.

35   In the case of gene transfer to 293 cells, pGUS plasmid-
directed β-glucuronidase expression exceeded expression

observed following GV10 UTV-vector mediated transfer of a
cis-linked reporter gene.

These results confirm that a vector comprising a
chimeric coat protein such as a chimeric fiber protein
5   according to the invention demonstrates increased
transfer of a nucleic acid that is not located in *cis*
with the vector. Ostensibly, this enhanced gene transfer
is effected by the occurrence of a protein/DNA
interaction between the negatively charged residues on
10   the chimeric fiber (e.g., residues of the polylysine
string), resulting in binding to the vector of the
nucleic acid; however, other means of enhancement also
are possible.

15   Example 7

This example describes the construction of further
plasmids containing UTV or UTV-like sequences in the C-
terminus of the fiber protein.

The transfer plasmid, p193(F5*) (Figure 12; also
20   known as p193NS (F5*), pNS (F5*), and pAd NS 83-100 UTV)
described in Example 2 was employed as a starting point
for the construction of these further plasmids containing
chimeric adenovirus fiber proteins. As depicted in
Figure 12, p193 (F5*) contains a mutated fiber gene with
25   a *Bam*HI site between the last fiber protein codon and the
frameshifted fiber protein stop codon. The further
mutant transfer plasmids constructed as described herein
contain sequences in the fiber C-terminus encoding an
amino acid glycine/serine repeat linker, a targeting
30   sequence, and a stop codon. These plasmids were made by
cloning synthetic oligonucleotides into the *Bam*HI site of
p193(F5*) to create the transfer plasmid p193NS (F5*)
pGS(K7)(also known as p193 (F5*) pGS(K7) or pNS (F5*)
pK7) depicted in **Figure 13**.
35   Thus, the sequence of the wild-type Ad5 fiber gene
is:

TCA TAC ATT GCC CAA GAA TAA AAA AGAA   [SEQ ID NO:59]
Ser Tyr Ile Ala Gln Glu   [SEQ ID NO:60]

wherein "TAA" is a termination codon, and the
polyadenylation sequence is emboldened. The C-terminus
of the mutated fiber gene present in p193(F5*) is:

5   TCA TAC ATT GCC CAA GAA <u>GGA TCC</u> **AATAAA** GAA   [SEQ ID NO:19]
    Ser Tyr Ile Ala Gln Glu Gly Ser   [SEQ ID NO:20]

wherein the underlined sequence indicates the mutated
10 BamHI site introduced into the fiber protein, and the
polyadenylation sequence is emboldened. In comparison,
the amino acid sequence of the C-terminus of the fiber
gene present in p193NS (F5*) pGS(K7) is:

15 <u>G S</u> G S G S G S G S **KKKKKK**   [SEQ ID NO:22]

wherein the underlined sequence indicates the mutated
BamHI site introduced into the fiber protein, and the
emboldened sequence indicates the polylysine string added
20 to the C-terminus. This amino acid sequence is encoded
by the nucleid acid sequence: GGA TCA GGA TCA GGT TCA GGG
AGT GGC TCT AAA AAG AAG AAA AAG AAG AAG TAA [SEQ ID
NO:21], wherein "TAA" is a termination codon.

The overlapping synthetic oligonucleotides used to
25 make the transfer plasmid p193NS (F5*) pGS(K7) were:
pK7s (sense), GA TCA GGA TCA GGT TCA GGG AGT GGC TCT AAA
AAG AAG AAA AAG AAG AAA TAA G [SEQ ID NO:61]; pK7a
(antisense), GA TCC TTA CTT CTT CTT TTT CTT CTT TTT AGA
GCC ACT CCC TGA ACC TGA TCC T [SEQ ID NO:62]. The sense
30 and antisense oligonucleotides were mixed in equimolar
ratios and cloned into the BamHI site of p193NS (F5*) to
create p193NS (F5*) pGS(pK7). Verification of the
correctly-oriented insert in p193NS (F5*) pGS(pK7) was
performed by PCR using the pK7s sense primer and the
35 downstream antisense oligonucleotide primer A5a32938,
CAGGTTGAATACTAGGGTTCT [SEQ ID NO:63]. The plasmid was
also verified to contain the correctly oriented insert by
sequencing the DNA sequence in the region of the insert
using the A5a32938 primer.

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The transfer plasmid p193NS (F5*) was employed in the construction of further mutant transfer plasmids that additionally contain a UTV or UTV-like cell targeting sequence in the C-terminus of the fiber protein. These
5   plasmids include p193NS (F5*) pGS(null) (also known as p193 (F5*) pGS(null) or p193 (F5*) pGS), pBSS 75-100 pGS (null), pBSS 75-100 pGS(RK32), pBSS 75-100 pGS(RK33), and pBSS 75-100 pGS(tat).

To construct p193NS (F5*) pGS(null), the
10   complementary overlapping oligonucleotides pGSs, GATCCGGTTCAGGATCTGGCAGTGGCTCGACTAGTTAAA [SEQ ID NO:64], and pGSa, GATCTTTAACTAGTCGAGCCACTGCCAGATCCTGAACCG [SEQ ID NO:65] were constructed for direct ligation into the BamHI-digested p193NS (F5*) plasmid. Verification of the
15   correctly-oriented clone was performed by PCR using the pGSs primer and the downstream antisense oligonucleotide primer A5a32938. The plasmid was also verified to contain the correctly oriented insert by sequencing the DNA sequence in the region of the insert using the
20   A5a32938 primer.

The vector pBSS 75-100 pGS(null) (also known as pBSS 75-100 ΔE3 pGS(null)) depicted in **Figure 14** was constructed by replacing the NheI to SalI fragment from pBSS 75-100 with the corresponding fragment from p193NS
25   (F5*) pGS(null). The SpeI site that is not within the fiber chimera gene was then eliminated by partially restricting the plasmid with SpeI, filling in with Klenow fragment and then religating the vector. The resultant vector comprises the relevant nucleic acid sequence:
30   GCCCAAGAAGGATCCGGTTCAGGATCTGGCAGTGGCTCGACTAGTTAA [SEQ ID NO:23] (wherein "TAA" is a termination codon), which codes for the amino acid sequence Ala Gln Glu Gly Ser Gly Ser Gly Ser Gly Ser Gly Ser Thr Ser [SEQ ID NO:24].
35   The inserts of plasmids pBSS 75-100 pGS(RK32) (also known as pBSS 75-100 ΔE3 pGS(RKKK)₂ or pBSS 75-100 ΔE3 pGS(RKKK2)), pBSS 75-100 pGS(RK33) (also known as pBSS

75-100 ΔE3 pGS(RKKK), or pBSS 75-100 ΔE3 pGS(RKKK3)), and
pBSS 75-100 pGS(tat), were constructed for direct
ligation into the *Spe*I-digested pBSS 75-100 pGS(null)
plasmid. To construct pBSS 75-100 pGS(RK32) depicted in
5   **Figure 15**, the complementary overlapping
oligonucleotides, RK32s, CTAGAAAGAAGAAACGCAAAAAGAAGA [SEQ
ID NO:66] and RK32a, CTAGTCTTCTTTTTGCGTTTCTTCTTT [SEQ ID
NO:67] were employed. The resultant vector comprises the
relevant nucleic acid sequence:
10 GCCCAAGAAGGATCCGGTTCAGGATCTGGCAGTGGCTCGACTAGAAAGAAGAAACGC
AAAAAGAAGACTAGTTAA [SEQ ID NO:25] (wherein "TAA" is a
termination codon), which codes for the amino acid
sequence Ala Gln Glu Gly Ser Gly Ser Gly Ser Gly Ser Gly
Ser Thr Arg Lys Lys Lys Arg Lys Lys Lys Thr Ser
15 [SEQ ID NO:26].

To construct pBSS 75-100 pGS(RK33) depicted in
Figure 16, the complementary overlapping
oligonucleotides, RK33s,
CTAGAAGAAGAAGCGCAAAAAAAAAAGAAAGAAGAAGA [SEQ ID NO:68]
20 and RK33a, CTAGTCTTCTTCTTTCTTTTTTTTTTGCGCTTCTTCTTCTTT
[SEQ ID NO:69] were employed. The resultant vector
comprises the relevant nucleic acid sequence:
GCCCAAGAAGGATCCGGTTCAGGATCTGGCAGTGGCTCGACTAGAAAGAAGAAGCGC
AAAAAAAAAGAAAGAAGAAGACTAGTTAA [SEQ ID NO:27] (wherein
25 "TAA" is a termination codon), which codes for the amino
acid sequence Ala Gln Glu Gly Ser Gly Ser Gly Ser Gly Ser
Gly Ser Thr Arg Lys Lys Lys Arg Lys Lys Lys Arg Lys Lys
Lys Thr Ser [SEQ ID NO:28].

To construct pBSS 75-100 pGS(tat) the complementary
30 overlapping oligonucleotides, TATs, CT AGT TAT GGG AGA
AAA AAG CGC AGG CAA CGA AGA CGG GCA T [SEQ ID NO:70] and
TATa, CT AGA TGC CCG TCT TCG TTG CCT GCG CTT TTT TCT CCC
ATA A [SEQ ID NO:71] were employed. The resultant vector
comprises the relevant nucleic acid sequence: ACT AGT TAT
35 GGG AGA AAA AAG CGC AGG CAA CGA AGA CGG GCA TCT AGT [SEQ
ID NO:72], which codes for the amino acid sequence Thr

Ser Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Ala Ser
Ser [SEQ ID NO:73].

Verification of the correctly-oriented clone was
performed by PCR using the sense primers (RK32s, RK33s,
5   or TATs) for each of the three respective plasmids, and
using the downstream antisense oligonucleotide primer
A5a32938. Each of the plasmids also were verified to
contain the correctly-oriented insert by sequencing the
DNA sequence in the region of the insert using the
10 A5a32938 primer.

                           Example 8
     This example described the construction of plasmids
containing UTV domains in the fiber loop.
15   Plasmids containing a UTV sequence (and/or a spacer
sequence) in an exposed loop of the fiber protein are
constructed by incorporating any of the aforesaid
sequences (as well as any further UTV-like sequences)
into the fiber protein. This is accomplished making use
20 of the plasmid transfer vector p193NS (F5*) to construct
the further transfer vector p193NS F5F2K (also called
p193 F5F2K) depicted in **Figure 17**. Plasmid p193NS F5F2K
contains a unique *Spe* I restriction site within the Ad2
fiber gene encoding an exposed loop in the protein.
25 Namely, the fiber gene present in p193NS F5F2K comprises
the fiber sequence:
ATT ACA CTT AAT GGC <u>ACT AGT</u> GAA TCC ACA
Ile Thr Leu Asn Gly Thr Ser Glu Ser Thr

30 GAA ACT
    Glu Thr .   [SEQ ID NO:29]
                                                 [SEQ ID NO:30]
wherein the underlined sequence indicates the novel *Spe* I
site introduced into the fiber gene.
     This vector was then used to clone targeting
35 sequences into the *Spe* I site. In particular, a nucleic
acid sequence encoding the stretch of 8 basic amino acids
RKKKRKKK (Arg Lys Lys Lys Arg Lys Lys Lys [SEQ ID NO:74])
comprising the heparin binding domain were cloned into

the *Spe* I site of p193 F5F2K using overlapping sense and
antisense oligonucleotides.

　　　　Namely, the (RKKK)₂ sequence comprises, in part, the
sequence:

5　　TCT AGA AAA AAA AAA CGC AAG AAG AAG ACT
　　　Thr Arg Lys Lys Lys Arg Lys Lys Lys Thr

　　　AGT   [SEQ ID NO:75]
　　　Ser   [SEQ ID NO:76].
10　　　The 27-mer sense oligonucleotide RK32s and 27-mer
antisense oligonucleotide RK32a described in Example 7
were employed for cloning the PolyGS(RKKK)₂ sequence
comprising the RKKKRKKK [SEQ ID NO:74] peptide motif.
The p193NS F5F2K(RKKK)₂ plasmid was constructed by cloning
15　　the DNA sequence encoding the binding domain into the *Spe*
I site of p193NS F5FK2. The overlapping sense and
antisense oligonucleotides encoding the binding domain
were first annealed and then directly ligated into the
Spe I restriction site to result in the plasmid p193NS
20　　F5F2K(RKKK)₂ depicted in Figure 18. This plasmid also is
known as p193NS F5F2K(RKKK2), p193NS F5F2K(RK32), or p193
F5F2K(RKKK2). The relevant portion of the modified loop
of the fiber knob present in p193NS F5F2K(RKKK)₂ is:

　　　ATT ACA CTT AAT GGC ACT AGA AAG AAG AAA CGC AAA AAG AAG
25　　Ile Thr Leu Asn Gly Thr Arg Lys Lys Lys Arg Lys Lys Lys

　　　ACT AGT GAA TCC ACA GAA ACT   [SEQ ID NO:31]
　　　Thr Ser Glu Ser Thr Glu Thr   [SEQ ID NO:32].
　　　　Furthermore, a (RKKK)₃ sequence, or other variations
30　　of this sequence, can be inserted into p193NS F5F2K.
This sequence comprises, in part:
　　　TCT AGA AAG AAG AAG CGC AAA AAA AAA AGA AAG AAG AAG
　　　Thr Arg Lys Lys Lys Arg Lys Lys Lys Arg Lys Lys Lys

35　　ACT AGT   [SEQ ID NO:77]
　　　Thr Ser   [SEQ ID NO:78].
The sequence can be inserted with use of the 39-mer sense
oligonucleotide (RKKK)₃(s) (i.e., comprising the sequence
CT AGA AAG AAG AAG CGC AAA AAA AAA AGA AAG AAG AAG A [SEQ
40　　ID NO:79]), and the 39-mer antisense oligonucleotide
(RKKK)₃(a) (i.e., comprising the sequence CT AGT CTT CTT
CTT TCT TTT TTT TTT GCG CTT CTT CTT T [SEQ ID NO:80]).

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The resultant plasmid p193NS F5F2K(RKKK), is depicted in **Figure 19**. This plasmid also is known as p193NS F5F2K(RKKK3), p193 F5F2K(RKKK3), or p193 F5FK(RK33). The relevant portion of the modified loop of the fiber knob present in p193 F5F2K(RKKK), is:

```
5   CTT AAT GGC ACT AGA AAG AAG AAG CGC AAA AAA AAA AGA AAG
    Leu Asn Gly Thr Arg Lys Lys Lys Arg Lys Lys Lys Arg Lys

    AAG ACT AGT GAA TCC ACA                    [SEQ ID NO:33]
10  Lys Thr Ser Glu Ser Thr                    [SEQ ID NO:34].
```

Example 9

This example describes the construction of plasmids containing chimeric penton base proteins comprising UTV or UTV-like sequences.

The transfer plasmid pACT (ΔRGD) (also described as plasmid pAT in U.S. Patent 5,559,099) was derived, in part, by manipulating a plasmid containing the unique *BamHI/PmeI* fragment (13259-21561) of the Ad5 genome, and contains, among other things, a penton base protein comprising a deletion of 8 amino acids constituting the α_v integrin binding domain, and a substitution of the deleted region for amino acids constituting a unique *SpeI* site, for the convenient insertion of exogenous sequences.

To construct plasmid pACT (RKKK), (also known as pACT (RKKK3) or pACT (RK33)) depicted in **Figure 20**, the complementary overlapping oligonucleotides RK33s and RK33a were directly ligated into a *SpeI*-digested pACT (ΔRGD) plasmid. Verification of the correctly-oriented clone was performed by PCR using the RK33a primer for the plasmid and the upstream sense oligonucleotide primer A5s15002. The plasmid also was verified to contain the correctly oriented insert by sequencing the DNA sequence in the region of the insert using the A5s15002 primer. The relevant portion of the UTV domain that will result in the chimeric penton base protein in pACT (RKKK), is:

```
AAC GAT ACT AGA AAG AAG AAG CGC AAA AAA AAA AGA AAG AAG AAG
```

Asn Asp Thr Arg Lys Lys Lys Arg Lys Lys Lys Arg Lys Lys Lys

ACT AGT GCC ACA   [SEQ ID NO:35].
Thr Ser Ala Thr   [SEQ ID NO:36].

5   The plasmid pACT (RK32) (which also can be called
pACT (RKKK2) or pACT (RKKK)₂) depicted in **Figure 21**
similarly can be constructed using the RK32s and RK32a
overlapping primers. The relevant portion of the UTV
domain present in the chimeric penton base protein in
10   pACT (RKKK)₂ is:

AAC GAT ACT AGA AAG AAG AAG AGA AAG AAG AAG ACT
Asn Asp Thr Arg Lys Lys Lys Arg Lys Lys Lys Thr

AGT GCC ACA   [SEQ ID NO:37]
15   Ser Ala Thr   [SEQ ID NO:38].

Example 10

     This example describes the construction of plasmids
containing UTV or UTV-like sequences in the adenovirus
20   hexon protein, and particularly which contain these
sequences in an exposed loop of the adenovirus hexon
protein.

     These plasmids can be constructed making use of
another transfer plasmid, plasmid pACT H11, depicted in
25   **Figure 22.** Plasmid pACT H11 itself is derived from
plasmid pACT (comprising from 13259-21561 of the Ad5
genome), which contains the majority of the hexon protein
coding sequence (corresponding to about 18842-21700). In
particular, pACT H11 can be constructed by incorporating
30   an *Xba*I site into the loop 1 region of the Ad5 hexon
protein. Similar techniques can be used to incorporate
an *Xba*I site, or any other convenient restriction site,
into either the loop 1 or the loop 2 region, or into
another exposed loop of the hexon protein. Sense and
35   antisense primers can be used to amplify the loop 1
region from Ad5 DNA by PCR, and at the same time
introduce a mutation which results in a unique mutated
*Xba*I site in the loop 1 region.
In particular, the sense primer,
40   GGACAGGGGCCCTACTTTTAAGCCCTACTCTGGCA [SEQ ID NO:81],

containing the naturally occurring unique restriction
site *ApaI* that occurs in pACT, and the antisense primer,
ATCTTCACTGTACAATACCACTTTAGGAGTCAAGTTATCACCTCTAGATGCGGTCGC
CT [SEQ ID NO:82], containing the unique restriction
site, *BsrGI*, can be employed. The PCR product,
containing the *XbaI* site can then be cut with *BsrGI* and
ApaI, and cloned back in to pACT to replace the *ApaI* to
BsrGI fragment. The resultant plasmid, pACT H11, contains
a unique *XbaI* site for the insertion of UTV sequences
into loop 1 of the hexon. The presence of the *XbaI* site
in the pACT H11 clone can be verified by restriction
digestion using *XbaI*, which should linearize the plasmid.

Part of the unmutated hexon loop 1 amino acid
sequence comprises the sequence TEATGNGDNL [SEQ ID
NO:83]. In comparison, the mutated hexon loop 1 amino
acid sequence following the wild-type TEA residues in
pACT H11 (**Figure 22**) comprises the sequence TASRGDNL [SEQ
ID NO:40] (i.e., encoded by the nucleic acid sequence
ACCGCATCTAGAGGTGATAACTTG [SEQ ID NO:39]).

The *XbaI* site of pACT H11 then can be used used as a
unique site in which to clone universal targeting
sequences such as RKKKRKKK [SEQ ID NO:74], for instance,
using the overlapping oligonucleotides, RK32s and RK32a.
The particular plasmid that results from such
manipulations, i.e. pACT H11 (RKKK)₂ (or pACT H11 (RK32)
or pACT H11 (RKKK2)) is depicted in **Figure 23**. This
plasmid comprises the sequence:

```
ACC GCA TCT AGA AAG AAG AAA CGC AAA AAG AAG ACT AGA
Thr Ala Ser Arg Lys Lys Lys Arg Lys Lys Lys Thr Arg

GGT GAT AAC TTG                                      [SEQ ID NO:41]
Gly Asp Asn Leu                                      [SEQ ID NO:42].
```

Other UTV or UTV-like sequences also can be cloned
in the loop 1 region of the hexon protein, and/or into
the loop 2 region of the hexon protein. For instance, a
similar approach can be used to mutate the sequence
encoding the hexon loop 2 to make plasmid pACT H12 (not
shown) that contains a unique restriction site (such as a

65

*Xba*I site) into which further UTV sequences can be
cloned.

Also, plasmid pACT H11 (RKKK)₃ (or pACT H11 (RKKK3)
or pACT H11 (RK33)) can be constructed making use of the
5   complementary overlapping oligonucleotides RK33s and
RK33a, and directly ligating the PCR product into the
*Xba*I-digested pACT H11 plasmid. Verification of the
correctly-oriented clone can be performed by PCR using
the RK32 sense primer for the plasmid and an appropriate
10 downstream antisense oligonucleotide primer. The plasmid
can be verified to contain the correctly oriented insert
by sequencing the DNA sequence in the region of the
insert using the downstream antisense primer. Similar
approaches can be employed for construction of analogous
15 transfer vectors, particularly the analogous transfer
vectors pACT H12 (RKKK)₂ and pACT H12 (RKKK)₃.

Example 11

This example describes the construction of a plasmid
20 having a short-shafted fiber protein. In particular,
this example describes the construction of the plasmid,
p193 F5F9sK.

The plasmid p193 F5F9sK (also known as p193 F5F9K-
Short) is depicted in **Figure 24**. This vector encodes a
25 chimeric fiber protein wherein approximately two thirds
of the Ad5 fiber shaft is deleted and the Ad5 fiber knob
is replaced with the Ad9 fiber knob.

The plasmid p193F5F9K-short was constructed from
p193NS (F5*). The oligonucleotide primers GGACTAGTAG
30 CATTTAATAA AAAAGAAGAT AAGCGC [SEQ ID NO:84] and
CCGGATCCTC ATTCTTGGGC GATATAGG [SEQ ID NO:85] were used
to amplify the Ad9 sequence encoding the last shaft
repeat and knob from the fiber gene. The PCR product was
then purified, using standard techniques, and digested
35 with the restriction enzymes *Nhe*I and *Bam*HI, which
allowed cloning of the PCR product into the *Nhe*I/*Bam*HI
region of the p193NS (F5*) transfer plasmid. The

resultant short-shafted fiber protein can be employed in construction of adenoviral vectors, as described below. Furthermore, any one or more of the aforementioned UTV or UTV-like sequences can be incorporated into the short-
5   shafted fiber, and the resultant fiber can be employed for cell delivery.

Example 12

        This example describes the construction of plasmids
10   containing UTV or UTV-like sequences in an extended structure, particularly in hexon and/or penton base protein, so as to result in lengthened hexon and/or penton base proteins that accordingly are better able to contact cells and participate in cell targeting. The
15   resultant chimeric proteins are "spiked" in the sense that they comprise an insertion of a nonnative amino acid sequence that will jut out from the virus surface.
        The primers 1alpha(s),
      GGGCTCCAGGCGGCCGCAGAAGCTGAAGAGGCAGCCACACGGGCTGAGGAGAA [SEQ ID
20   NO:86], and 1alpha(a),
      GGGGTGCACACAGCTTCGGCCTTAGCGTTAGCCTGTTTCTTCTGAGGCTTCTCGACCT
      [SEQ ID NO:87], can be used to amplify the region of the penton gene encoding the 32 amino acid α-helical domain that follows the RGD sequence. This 32 amino acid
25   sequence comprises the sequence
      ATRAEEDRAEAEAAAEAAAPAAQPEVEKPQKK [SEQ ID NO:88]. The
      primers used also can encode an additional α-helical sequence on either end, such that, for instance, the final amplified DNA sequence encodes the sequence:
30   CTG CAG GCG GCC GCA GAA GCT GAA GAG GCA GCC ACA CGG GCT GAG
      Leu Gln Ala Ala Ala Glu Ala Glu Glu Ala Ala Thr Arg Ala Glu

      GAG AAG CGC GCT GAG GCC GAA GCA GCG GCC GAA GCT GCC GCC CCC
      Glu Lys Arg Ala Glu Ala Glu Ala Ala Ala Glu Ala Ala Ala Pro
35
      GCT GCG CAA CCC GAC GTC GAG AAG CCT CAG AAG AAA CAG GCT AAC
      Ala Ala Gln Pro Glu Val Glu Lys Pro Gln Lys Lys Gln Ala Asn

      GCT AAG GCC GAA GCT GTG CAG GCG GCC GCA GAA GCT GAA GAG GCA
40   Ala Lys Ala Glu Ala Val Gln Ala Ala Ala Glu Ala Glu Glu Ala

```
GCC ACA CGG GCT GAG GAG AAG CGC GCT GAG GCC GAA GCA GCG GCC
Ala Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala Glu Ala Ala Ala

GAA GCT GCC GCC CCC GCT GCG CAA CCC GAG GTC GAG AAG CCT CAG
Glu Ala Ala Ala Pro Ala Ala Gln Pro Glu Val Glu Lys Pro Gln

AAG AAA CAG GCT AAC GCT AAG GCC GAA GCT GTG CAC
[SEQ ID NO:89]
Lys Lys Gln Ala Asn Ala Lys Ala Glu Ala Val His
[SEQ ID NO:90]
```

where the emboldened sequence corresponds to the
nonpenton sequence encoded by the primers, and the
underlined sequence represents the amino acids encoded by
the two compatible restriction sites, SfcI and ApaLI.
These amino acids also preserve the integrity of an alpha
helix according to standard computer programs designed to
predict α helix structure.

The PCR product encoding these amino acids can be
cut with both SfcI and ApaLI, religated, and then cut
again with both enzymes. Ligation of like-site to like-
site preserves the site for recutting; however, ligation
of the compatible, but unlike sites, destroys the site.
Therefore, upon recutting of the ligated product,
multiple fragments will be produced which are multiples
of the original size of the PCR product. There will be
completely recut fragments (approximately 150 bp),
approximately 300 bp fragments (having one restriction
site destroyed), and approximately 450 bp fragment
(having 2 sites destroyed), and so on. The procedure
accordingly allows the original sequence encoding 50
amino acids (1alpha) to be doubled (2alpha), tripled
(3alpha), and so on, for cloning large, uninterrupted α-
helical regions into a protein to create a larger
"spike", or extension of the protein.

For instance, the 2alpha double product (i.e.,
2alpha2) can be cloned into the first Ppu10I site of the
plasmid, pSPdelta (depicted in Figure 25), to create the
plasmid pSP2alpha (depicted in Figure 26). The plasmid
pSPdelta is constructed from the base plasmid pUC19, or
any other suitable cloning plasmid. The pSPdelta

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transfer plasmid can be employed to make further
modifications of the penton or hexon protein that allow
the UTV sequence (or any other targeting sequence) to be
elevated out from the virion surface. This elevation of

5   the targeting sequence in a spiked structure (e.g., a
type of "tower") will minimize steric hindrance of the
penton and hexon interaction with the cell surface by the
fiber protein, and will allow greater access of the
chimeric penton and hexon proteins for interacting with

10   the cell surface.

The plasmid pSPdelta contains a unique SpeI cloning
site for the incorporation of UTV sequences. The SpeI
cloning site is flanked on either side by Ppu10I cloning
sites which allow the incorporation of DNA sequences

15   encoding amino acids that will elevate the UTV sequence
away from the virion surface. The pSPdelta plasmid was
constructed by cloning into the unique XbaI site of pUC19
overlapping oligonucleotides which are designed to insert
directly into the XbaI site. In particular, the sense

20   oligonucleotide is:
CTAGAGCAGCTATGCATGAAGGGACTAGTGGAGAGATGCATGCAGCCT [SEQ ID
NO:91]. The antisense, complementary oligonucleotide is:
CTAGAGGCTGCATGCATCTCTCCACTAGTCCCTTCATGCATAGCTGCT [SEQ ID
NO:92]. The oligonucleotides are mixed in equimolar

25   ratios and cloned into the XbaI site of pUC19. The
presence of the correct insert can be confirmed by
sequencing across the region of the insert, and by
cutting the plasmid with Ppu10I. The XbaI sites on
either side of the insert allow the convenient removal of

30   this section in later clones.

Because there are two Ppu10I sites in pSPdelta, the
plasmid can be partially restricted upon digestion with
Ppu10I so that only a single site is cut. Ligation of
the PCR product comprising the ApaLI and SfcI sites into

35   the compatible Ppu10I site will destroy the first Ppu10I
site in the plasmid and also destroy the ApaLI and SfcI
sites. These destroyed sites are represented in **Figure**

69

26 (showiing the pSP2alpha2 plasmid) by a "j". A second
doublet product then can be cloned into the remaining
Ppu10I site of the pSP2alpha plasmid to produce the
plasmid pSP2alpha2 depicted in Figure 27, which contains

5   an *Spe*I site for insertion cloning of a UTV sequence, or
other similar targeting sequence. Computer analysis of
the secondary structure of the anticipated 2alpha2
protein confirms that it will be a complete α helix
except for in the region of the *Spe*I cloning site.

10   Thus, whereas pSPdelta comprises the sequence:
TCT AGA GCA GCT ATG CAT GAA GGG ACT AGT GGA GAC ATG CAT GCA
Ser Arg Ala Ala Met His Glu Gly Thr Ser Gly Glu Met His Ala

        GCC TCT AGA   [SEQ ID NO:43]
15   Ala Ser Arg   [SEQ ID NO:44],

        pSP2alpha comprises the sequence:

        TCT AGA GCA GCT ATG CAG GCG GCC GCA GAA GCT GAA GAG GCA
20   Ser Arg Ala Ala Met Gln Ala Ala Ala Glu Ala Glu Glu Ala

        GCC ACA CGG GCT GAG GAG AAG CGC GCT GAG GCT GAA GCA GCG
        Ala Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala Glu Ala Ala

25   GCC GAA GCT GCC GCC CCC GCT GCG CAA CCC GAG GTC GAG AAG
        Ala Glu Ala Ala Ala Pro Ala Ala Gln Pro Glu Val Glu Lys

        CCT CAG AAG AAA CAG GCT AAC GCT AAG GCC GAA GCT GTG CAG
        Pro Gln Lys Lys Gln Ala Asn Ala Lys Ala Glu Ala Val Gln
30
        GCG GCC GCA GAA GCT GAA GAG GCA GCC ACA CGG CCT GAG GAG
        Ala Ala Ala Glu Ala Glu Glu Ala Ala Thr Arg Ala Glu Glu

        AAG CGC GCT GAG GCC GAA GCA GCG GCC GAA GCT GCC GCC CCC
35   Lys Arg Ala Glu Ala Glu Ala Ala Ala Glu Ala Ala Ala Pro

        GCT GCG CAA CCC GAG GTC GAG AAG CCT GAG AAG AAA CAG GCT
        Ala Ala Gln Pro Glu Val Glu Lys Pro Glu Lys Lys Gln Ala

40   AAC GCT AAG GCC GAA GCT GTG CAT GAA GGG ACT AGT GGA GAG
        Asn Ala Lys Ala Glu Ala Val His Glu Gly Thr Ser Gly Glu

        ATG CAT GCA GCC TCT AGA   [SEQ ID NO:45]
        Met His Ala Ala Ser Arg   [SEQ ID NO:46],
45

        and pSP2alpha2 comprises the sequence:
        TCT AGA GCA GCT ATG CAG GCG GCC GCA GAA GCT GAA GAG GCA GCC
        Ser Arg Ala Ala Met Gln Ala Ala Ala Glu Ala Glu Glu Ala Ala

```
     ACA CGG GCT GAG GAG AAG CGC GCT GAG GCC GAA GCA GCG GCC GAA
     Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala Glu Ala Ala Ala Glu

  5  GCT GCC GCC CCC GCT GCC CAA CCC GAG GTC GAG AAG CCT CAG AAG
     Ala Ala Ala Pro Ala Ala Gln Pro Glu Val Glu Lys Pro Gln Lys

     AAA CAG GCT AAC GCT AAG GCC GAA GCT GTG CAG GCG GCC GCA GAA
     Lys Gln Ala Asn Ala Lys Ala Glu Ala Val Gln Ala Ala Ala Glu

 10  GCT GAA GAG GCA GCC ACA CGG GCT GAG GAG AAG CGC GCT GAG GCC
     Ala Glu Glu Ala Ala Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala

     GAA GCA GCG GCC GAA GCT GCC GCC CCC GCT GCG CAA CCC GAG GTC
     Glu Ala Ala Ala Glu Ala Ala Ala Pro Ala Ala Gln Pro Glu Val

 15  GAG AAG CCT CAG AAG AAA CAG GCT AAC GCT AAG GCC GAA GCT GTG
     Glu Lys Pro Gln Lys Lys Gln Ala Asn Ala Lys Ala Glu Ala Val

     CAT GAA GGG ACT AGT GGA GAG ATG CAG GCG GCC GCA GAA GCT GAA
     His Glu Gly Thr Ser Gly Glu Met Gln Ala Ala Ala Glu Ala Glu

 20  GAG GCA GCC ACA CGG GCT GAG GAG AAG CGC GCT GAG GCC GAA GCA
     Glu Ala Ala Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala Glu Ala

 25  GCG GCC GAA GCT GCC GCC CCC GCT GCG CAA CCC GAG GTC GAG AAG
     Ala Ala Glu Ala Ala Ala Pro Ala Ala Gln Pro Glu Val Glu Lys

     CCT CAG AAG AAA CAG GCT AAC GCT AAG GCC GAA GCT GTG CAG GCG
     Pro Gln Lys Lys Gln Ala Asn Ala Lys Ala Glu Ala Val Gln Ala

 30  GCC GCA GAA GCT GAA GAG GCA GCC ACA CGG GCT GAC GAG AAG CGC
     Ala Ala Glu Ala Glu Glu Ala Ala Thr Arg Ala Glu Glu Lys Arg

 35  GCT GAG GCC GAA GCA GCG GCC GAA GCT GCC GCC CCC GCT GCG CAA
     Ala Glu Ala Glu Ala Ala Ala Glu Ala Ala Ala Pro Ala Ala Gln

     CCC GAG GTC GAG AAG CCT CAG AAG AAA CAG GCT AAC GCT AAG GCC
     Pro Glu Val Glu Lys Pro Gln Lys Lys Gln Ala Asn Ala Lys Ala

 40  GAA GCT GTG CAT GCA GCC TCT AGA                [SEQ ID NO:47]
     Glu Ala Val His Ala Ala Ser Arg                [SEQ ID NO:48].
```

Targeting sequences such as UTV or UTV-like
sequences can be cloned into the *Spe*I site of the plasmid
45 pSP2alpha2. In particular, the RK32s and RK32a
overlapping oligonucleotides can be cloned into the *Spe*I
site to create pSP2alpha2 (RKKK)₂ (or, pSP2alpha2 (RK32)
or pSPSalpha2 (RKKK2)). The plasmid pSP2alpha2 (RKKK)₃
(or, pSP2alpha2 (RK33) or pSPSalpha2 (RKKK3)) can be
50 similarly constructed. Alternately, the entire 2alpha2
α-helical domain can be removed from the plasmid by
restriction with *Xba*I and cloned into the compatible *Spe*I
site of pACT (ΔRGD) to create pACT 2alpha2 (RKKK)₂ (which

also can be called pACT 2alpha2 (RKKK2) or pACT 2alpha2
(RK32)). Similar techniques can be employed to produce
pACT 2alpha2 (RKKK)₃ (which also can be called pACT
2alpha2 (RKKK3) or pACT 2alpha2 (RK33)).

5   Similarly, chimeric hexon proteins that are spiked
(i.e., comprise sequences resulting in their extension)
can be constructed by cloning the 2alpha2 α-helical
domain into the XbaI site of pACT H11 to create pACT H11
2alpha2 (RKKK)₂ (which also can be called pACT H11 2alpha2

10   (RKKK2) or pACT H11 2alpha2 (RK32)). Similar techniques
can be employed to produce pACT H12 2alpha2 (RKKK)₂ (which
also can be called pACT H12 2alpha2 (RKKK2) or pACT H12
2alpha2 (RK32)).

15   Example 13

      This example describes the construction of further
adenoviral vectors, in addition to those previously
described, which contain UTV or UTV-like sequences in the
adenoviral fiber protein.

20   Construction of adenovirus vectors containing UTV
modifications in the fiber can be accomplished in
multiple ways by those skilled in the art. One method to
create the UTV fiber vectors from plasmids described
above is to first linearize the plasmid DNA with SalI and

25   then transfect this DNA into a 293 packaging cell line
that was infected just prior to transfection with an E4-
deleted adenovirus. E4-deleted adenovirus vectors are
incapable of replicating in cell lines such as the 293
cell line, which only provide the adenovirus E1 regions

30   in trans. Recombination of the plasmids which contain
the modified fiber gene and the E4 regions with the E4-
deleted DNA results in a replication-competent, E4-
containing vector which carries the modified fiber gene.
      Accordingly, the plasmids p193NS (F5*) pGS(K7), pBSS

35   75-100 pGS(null), pBSS 75-100 pGS(RKKK)₂, pBSS 75-100
pGS(RKKK)₃, pBSS 75-100 pGS(tat), p193NS F5F2K(RK32), and
p193 F5F9sK were each linearized with SalI and

transfected into 293 cells infected 1 hour prior with
either the E4-deleted adenovirus vector, GV11A.Z (which
carries the LacZ gene under the control of a
cytomegalovirus (CMV) promoter), or GV11A.S (which

5   carries a secretory alkaline phosphatase gene under the
control of a CMV promoter). The resultant adenovirus
vectors, AdZ.F(pK7), AdZ.F(pGS), AdZ.F(RKKK)$_2$ (also known
as AdZ.F(RKKK2) or AdZ.F(RK32)), AdZ.F(RKKK)$_3$ (also known
as AdZ.F(RKKK3) or AdZ.F(RK33)), AdZ.F(tat),

10   AdZ.F5F2K(RKKK)$_2$ (also known as AdZ.F5F2K(RKKK2) or
AdZ.F5F2K(RK32)), and AdZ.F5F9sK (also known as
AdZ.F5F9K-Short) were obtained and were purified through
two successive rounds of plaquing on 293 cells.

       All the vectors were verified to contain the correct
15   sequence through PCR across the region of the insert, and
by restriction analysis of viral DNA obtained from
vector-infected 293 cells by Hirt extraction. Western
analysis (as previously described) also can be employed
to examine protein size, if so desired. Western analysis

20   of fiber protein from vector particles and/or vector-
infected cell lysates electrophoresed on a polyacryamide
gel should show a corresponding shift in the mobility of
the fiber protein compared to unmodified fiber protein
that is consistent with the presence of additional amino

25   acid sequences. For instance, Western analysis of
AdZ.F(pK7) particles verified that its fiber protein is
shifted up compared to that of the AdZ vector comprising
unmodified fiber, consistent with the presence of
additional amino acids in the AdZ.F(pK7) fiber protein.

30   Other plasmid maps depicted herein similarly can be
made into adenoviral vectors by utilizing the same
procedure outlined above (or minor variations thereof).

Example 14

35   This example describes the construction of
adenoviral vectors which contain UTV or UTV-like
sequences in the adenoviral penton base protein.

The method of making an adenoviral vector comprising a chimeric penton base protein is described, for instance, in Wickham et al., J. Virol., 70, 6831-6838 (1996). A pACT vector described above containing the
5   chimeric penton base protein (e.g., transfer plasmids pACT 2alpha2 (RKKK)₂, pACT H12 2alpha2 (RKKK)₂, and pACT (ΔRGD)) can be digested, for instance, with BamHI, to linearize the plasmid. Ad5 DNA can be digested with the restriction endonuclease XmnI, which cuts wild-type Ad5
10   at positions 14561 and 15710 within the Ad5 genome. The two larger fragments are purified away from the smaller 1 kb piece then transfected along with the linearized plasmid into the appropriate cell line (e.g., a 293 cell line) to produce recombinant virus.
15   Adenoviral vectors produced in this fashion are purified from potentially-contaminating unmodified vectors through two successive rounds of plaque purification on 293 cells. The resultant vectors are then verified to contain the correct sequence in the
20   penton base region through restriction analysis of viral DNA obtained following Hirt extraction of vector-infected 293 cells. Sequencing of PCR products generated by amplifying the region of the insert from the viral DNA can also be used to verify the presence of the insert.
25   Western analysis of the chimeric penton base electrophoresed on a polyacrylamide gel should show a corresponding shift in the mobility of the penton base compared to unmodified penton base that is consistent with the presence of additional amino acid sequences in
30   the chimeric protein.

Example 15

This example describes the construction of adenoviral vectors containing UTV or UTV-like sequences
35   in the hexon protein.
     To construct the virus AdZ.H(RKKK)₂, left and right vector arms are prepared that contain DNA sequences which

overlap and will recombine on either side of the *PmeI* to *BamHI* pACT H11(RK32) sequence to create an intact AdZ.H (RK32) genome. To construct the left arm, purified Ad5 DNA is restriction digested with *AgeI*, which cuts Ad5 at
5   positions 14499, 15283, 19017, 23063, 23656, 23411, and 31102. The 0-14499 fragment then can be employed as the left arm and purified from the other fragments by gel electrophoresis. The right arm can be prepared by digesting Ad5 DNA with *DrdI*. *DrdI* cuts Ad5 at positions
10 5458, 7039, 14004, 15593, 17257, and 21023. The 21023-35938 bp fragment then can be used as the right arm and purified from the other fragments by gel electrophoresis. These two fragments are then transfected with the *PmeI/BamHI* fragment from pACT H11(RK32) into 293 cells.
15 *PmeI* cuts at position 13258 in Ad5, and *BamHI* cuts at position 21562 in Ad5. Similar techniques can be employed to produce AdZ.H(RKKK)₃.

                              Example 16
20   This example describes the construction of vectors containing a short-shafted fiber protein.
         The method described herein for construction of an adenovirus from the transfer plasmid p193 F5F9Kshort can similarly be employed for the construction of other
25 adenoviral vectors from other short-shafted fibers. Namely, the transfer plasmid p193 F5F9Kshort, which contains the essential E4 region of adenovirus, was cut with *SalI* and transfected into 293 cells, which had been infected one hour earlier with the adenoviral vector
30 AdSE.E4Gus. AdSE.E4Gus lacks the E4 region of the adenoviral genome, and cannot replicate in 293 cells in the absence of complementation for the E4 genes. Thus, only when the AdSE.E4Gus DNA recombines with the p193 F5F9K short plasmid DNA to obtain the E4 genes is the
35 vector able to replicate in 293 cells. During this recombination event, the newly formed vector also acquires the mutated fiber protein coding sequences

encoded by the plasmids.   Viable recombinant E4*
adenovirus containing the F5F9Kshort fiber chimera were
then isolated by plaquing the transfected cell lysates 5
days after transfection.   The resultant vector
5   AdSE.F5F9Kshort was isolated and purified by standard
virological techniques involving two successive rounds of
plaquing on 293 cells.   The vector was verified to
contain the correct insert by PCR and restriction
analysis of viral DNA.   Oligonucleotide primers, which
10   prime on either side of the fiber gene, confirmed that
the PCR product was of the correct size for that encoded
by a shortened chimeric fiber gene.   Restriction analysis
of the vector DNA showed that the new vector contained
the correct restriction sites that are unique to the Ad9
15   fiber knob.

                        Example 17
         This example describes the construction of
adenoviral vectors that contain short-shafted fiber
20   proteins and chimeric penton base proteins incorporating
UTV or UTV-like sequences.
         For this construction, AdS.F9sK viral DNA can be
digested with XmnI, as described above.   The plasmid pACT
H11(RK32) is then cut with the restriction enzymes PmeI
25   and BamHI.   The restriction digested viral and plasmid
DNAs are purified and transfected into 293 cells.   The
resultant vectors are isolated by two successive rounds
of plaque purification on 293 cells, and are verified to
contain the correct sequence in the penton base region by
30   restriction analysis of viral DNA obtained from vector-
infected 293 cells by Hirt extraction.
         Sequencing of PCR products generated by amplifying
the region of the insert from the viral DNA also can be
used to verify the presence of the insert.   Western
35   analysis of the chimeric penton base protein on a
polyacrylamide gel should show a corresponding shift in
the mobility of the chimeric penton base compared to

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unmodified penton base that is consistent with the
presence of additional nonnative amino acid sequences
(and absence of native amino acid sequences).

Other plasmids for which maps are presented herein
and which were not made into an adenoviral vector can be
made by utilizing the same or a slightly modified version
of the procedure outlined above. In particular, the
short-shafted fiber protein can be incorporated into an
adenovirus having a "spiked" chimeric penton base protein
that furthermore optionally can incorporate a UTV or UTV-
like sequence.

Example 18

This example describes the construction of
adenoviral vectors that contain short-shafted fiber
proteins and chimeric hexon proteins incorporating UTV or
UTV-like sequences.

Viral DNA can be isolated from a short-shafted
vector such as AdZ.F9sK and cut with the restriction
enzymes described above for making vectors comprising
UTV-containing chimeric hexon proteins. All other steps
are the same as described, for instance, in Example 17.
The resultant vector should contain the short-shafted
fiber protein and the chimeric hexon protein
incorporating UTV or UTV-like sequences. Moreover, this
approach can be employed with a variety of transfer
plasmids comprising different chimeric hexon proteins.
In particular, the short-shafted fiber protein can be
incorporated into an adenovirus having a "spiked"
chimeric hexon protein that furthermore optionally can
incorporate a UTV or UTV-like sequence.

Example 19

This example describes an evaluation of vectors,
particularly adenoviral vectors, according to the
invention, which comprise UTV or UTV-like sequences.

For instance, to confirm that the addition of UTV or
UTV-like sequences has no effect on virus assembly, the
virus growth kinetics of the vectors can be assessed. As
representative of a UTV-sequence containing plasmid, the
5   growth behaviour of pAd.F(pK7) was monitored and compared
to that of wild-type adenovirus (Ad5), as well as the
adenoviral vector AdZ.F(RGD), which contains an insertion
of a RGD peptide motif present in the sequence
SACDCRGDCFCGTS [SEQ ID NO:93]. For these studies, 293
10 cells were infected at a multiplicity of infection of 5
active virus particles/cell with either Ad5, AdZ.F(RGD)
or AdZ.F(pK7), and the number of infectious particles
(fluorescent focus units (FFU)) produced per cell was
determined following the harvesting of the cells at 1, 2,
15 and 3 days post infection. The titers of AdZ.F(RGD) or
AdZ.F(pK7) were somewhat lower, but not dramatically
different than the titer of Ad5. As can be seen in
Figure 28, the peak titers of AdZ.F(RGD) and AdZ.F(pK7)
were 80% and 56%, respectively, that of Ad5. These
20 results confirm that the growth kinetics of the two
vectors are not substantially affected by the addition of
sequences, particularly a UTV or UTV-like sequence, onto
the end of the fiber protein. The results also suggest
that further vectors comprising UTV or UTV-like sequences
25 will not exhibit aberrant growth behaviour.
        Furthermore, vectors containing UTV or UTV-like
sequences can be evaluated for their ability to bind to
cells or deliver genes to be inhibited by negatively-
charged molecules (e.g., heparin, heparan sulfate,
30 chondroitan sulfate, etc.), soluble adenoviral coat
proteins, or by pretreatment of cells with agents (e.g.,
chondroitinase, heparinase, sialidase, etc.) that cleave
such negatively-charged moieties (see, e.g., Wickham et
al., Nature Biotechnology, 14, 1570-1573 (1996), as well
35 as the preceding Examples). Soluble fiber protein will
not inhibit the majority of the binding of a UTV vector
to a cell (Wickham et al. (1996), supra). These results

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suggest that the incorporation of UTV or UTV-like sequences into penton, hexon or fiber will not impair the ability of a recombinant adenovirus containing the chimeric coat protein to effect gene delivery.

5   Also, studies of infection *in vivo*, or *in vitro* transfections done in the presence of whole blood, can be employed to confirm that the UTV vectors of the present invention are not limited for systemic delivery due to saturation of the polycations on the recombinant

10   adenoviruses with polyanions in the blood. In the event that such binding impedes the capability of a particular virus for target cell transduction, the virus can be administered in a higher dose, preferably with provision being made to reduce any immune response associated with

15   such a higher dose (e.g., administration of another serotype of adenoviral vector, or techniques described in PCT International Application WO 96/12406; Mastrangeli et al., Human Gene Therapy, 7, 79-87 (1996)).

20   All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference to the same extent as if each reference were set forth in its entirety herein.

25   While this invention has been described with an emphasis upon preferred embodiments, it will be apparent to those of ordinary skill in the art that variations in the preferred embodiments can be prepared and used and that the invention can be practiced otherwise than as

30   specifically described herein. The present invention is intended to include such variations and alternative practices. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

35

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: GenVec, Inc.

    (ii) TITLE OF INVENTION: VECTORS AND METHODS FOR GENE TRANSFER TO
        CELLS

    (iii) NUMBER OF SEQUENCES: 12

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Leydig, Voit & Mayer, Ltd.
        (B) STREET: Two Prudential Plaza, Suite 4900
        (C) CITY: Chicago
        (D) STATE: IL
        (E) COUNTRY: USA
        (F) ZIP: 60601

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: PCT
        (B) FILING DATE: 27-NOV-1996

    (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 08/563,368
        (B) FILING DATE: 28-NOV-1995
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (C) REFERENCE/DOCKET NUMBER: 74862

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    Lys Lys Lys Lys Lys Lys Lys Lys
    1   5

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Arg Arg Arg Arg Arg Arg Arg Arg
1   5

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (C) OTHER INFORMATION: Xaa is Lys or Arg

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1   5

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Ser Asn Lys Glu Ser Phe Val Leu Lys Lys Lys Lys Lys Lys
1   5   10   15

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Gly Ser Asn Lys Asn Lys Glu Ser Phe Val Leu Lys Lys Lys Lys
1   5   10   15

Lys Lys

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8 base pairs
        (B) TYPE: nucleic acid

```
                    (C) STRANDEDNESS: double
                    (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: DNA (genomic)



            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGA TCC AA                                                              8
Gly Ser
  1
```

```
    (2) INFORMATION FOR SEQ ID NO:7:

            (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 30 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: double
                    (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: DNA (genomic)



            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGA TCC AAT AAA GAA TCG TTT GTG TTA TGT                                30
Gly Ser Asn Lys Glu Ser Phe Val Leu Cys
  1               5                   10
```

```
    (2) INFORMATION FOR SEQ ID NO:8:

            (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 36 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: double
                    (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: DNA (genomic)



            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCC GGA TCC AAC AAG AAT AAA GAA TCG TTT GTG TTA                        36
Ala Gly Ser Asn Lys Asn Lys Glu Ser Phe Val Leu
  1               5                   10
```

```
    (2) INFORMATION FOR SEQ ID NO:9:

            (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 55 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: DNA (synthetic)


            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TATGGAGGAT CCAATAAAGA ATCGTTTGTG TTATGTTTCA ACGTGTTTAT TTTTC          55
```

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(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 57 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (synthetic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AATTGAAAAA TAAACACGTT GAAACATAAC ACAAACGATT CTTTATTGGA TCCTCCA   57

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 43 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (synthetic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCCCCCCGGG TCTAGATTAG GATCCTTCTT GGGCAATGTA TGA   43

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (synthetic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGTGTATCCA TATGACACAG A   21

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WHAT IS CLAIMED IS:

1.   A chimeric adenovirus coat protein comprising a
nonnative amino acid sequence, wherein said chimeric
adenovirus coat protein is able to direct entry into
cells of a vector comprising said chimeric adenovirus
coat protein that is more efficient than entry into cells
of a vector that is identical except for comprising a
wild-type adenovirus coat protein rather than said
chimeric adenovirus protein.

2.   The chimeric adenovirus coat protein of claim
1, wherein said chimeric adenovirus coat protein binds a
novel binding site present on the cell surface.

3.   The chimeric adenovirus coat protein of claim 1
or 2, wherein said nonnative amino acid sequence is
inserted into or in place of an internal coat protein
sequence.

4.   The chimeric adenovirus coat protein of claim 1
or 2, wherein said nonnative amino acid sequence is at or
near the C-terminus of said protein.

5.   The chimeric adenovirus coat protein of claim 1
or 2, wherein said nonnative amino acid sequence is in an
exposed loop of said protein.

6.   The chimeric adenovirus coat protein of any of
claims 1-5, wherein said nonnative amino acid sequence
comprises a UTV or UTV-like sequence which is linked to
said protein by a spacer sequence of from about 3 to
about 400 amino acids.

7.   The chimeric adenovirus coat protein of claim
6, wherein said spacer sequence is selected from the
group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID

19.   The nucleic acid of claim 16, wherein said chimeric adenovirus coat protein is a hexon protein.

20.   A vector that comprises a chimeric adenovirus coat protein of any of claims 1-15.

21.   The vector of claim 20, wherein said vector is a viral vector selected from the group consisting of nonenveloped viruses.

22.   The vector of claim 20, wherein said vector is an adenoviral vector.

23.   The vector of any of claims 20-22, wherein said vector further comprises a nucleic acid comprising a passenger gene.

24.   The vector of claim 23, wherein said nucleic acid is inserted into the adenoviral genome.

25.   The vector of claim 23, wherein said nucleic acid is attached to said chimeric adenovirus coat protein by means of a protein/DNA interaction.

26.   A vector selected from the group consisting of GV10 UTV, AdZ.H(RKKK)$_2$, AdZ.H(RKKK)$_3$, AdZ.F(RKKK)$_2$, AdZ.F(RKKK)$_3$, AdZ.F(pK7), AdZ.F(pGS), AdZ.F(tat), AdZ.F52K(RKKK)$_2$, and AdZ.F52K(RKKK)$_3$.

27.   The vector of any of claims 20-25, wherein said chimeric adenovirus coat protein is a fiber protein.

28.   The vector of any of claims 20-25, wherein said chimeric adenovirus coat protein is a penton base or hexon protein.

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29.   The vector of claim 28, wherein said vector does not comprise wild-type fiber protein.

30.   The vector of claim 28, wherein said vector comprises a short-shafted fiber protein.

31.   A transfer vector that encodes the chimeric adenovirus coat protein of any of claims 1-15.

32.   The transfer vector of claim 31, wherein said transfer vector is selected from the group consisting of nonenveloped viruses.

33.   The transfer vector of claim 31, wherein said transfer vector is an adenoviral vector.

34.   The transfer vector of any of claims 31-33, wherein said transfer vector further comprises a nucleic acid encoding a passenger gene.

35.   A transfer vector selected from the group consisting of p193 (F5*), pAd BS 59-100 UTV, p193NS (F5*) pGS(pK7), p193NS (F5*) pGS(null), pBSS 75-100 pGS (null), pBSS 75-100 pGS(RK32), pBSS 75-100 pGS(RK33), pBSS 75-100 pGS(tat), p193 F5F2K, p193 F5F2K(RKKK)$_2$, p193 F5F2K(RKKK)$_3$, pACT (RKKK)$_3$, pACT (RKKK)$_2$, pACT H11, pACT H11 (RKKK)$_2$, pACT H11 (RKKK)$_3$, pACT H12, pACT H12 (RKKK)$_2$, pACT H12 (RKKK)$_3$, pSP2alpha, pSP2alpha2, pSP2alpha2(RKKK)$_2$, pSP2alpha2(RKKK)$_3$, pACT2alpha2 (RKKK)$_2$, pACT2alpha2 (RKKK)$_3$, pACT H11 2alpha2 (RKKK)$_2$, pACT H11 2alpha2 (RKKK)$_3$, pACT H12 2alpha2 (RKKK)$_2$, and pACT H12 2alpha2 (RKKK)$_3$.

36.   The transfer vector of any of claims 31-34, wherein said coat protein is a fiber protein.

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37. The transfer vector of any of claims 31-34, wherein said coat protein is a penton base or hexon protein.

5   38. A method of increasing the efficiency of entry into cells of a vector comprising a coat protein, which comprises replacing a coat protein of said vector with the chimeric adenovirus coat protein of any of claims 1-15.

10

39. A method of genetically modifying a cell which comprises contacting said cell with a vector of any of claims 20-37.

15   40. A method of genetically modifying a cell which comprises:
         (a) obtaining a vector comprising the coat protein of any of claims 1-15,
         (b) contacting said vector with a nucleic acid
20   encoding a passenger gene such that said chimeric protein and said nucleic acid bind due to a protein/DNA interaction, and
         (c) contacting said cell with said vector comprising bound nucleic acid.

25

41. A host cell comprising a vector of any of claims 20-37.

42. A method of making the chimeric adenovirus coat
30   protein of claim 1, which method comprises introducing a spacer sequence into the coding sequence of said chimeric adenovirus coat protein prior to any stop codon or polyadenylation signal.

35   43. The method of claim 42, wherein said spacer sequence comprises the sequence of SEQ ID NO:6 or a conservatively modified variant thereof.

44.   A method of making the vector GV10 UTV, wherein
said method comprises providing to a cell that does not
comprise any E4 complementing sequences: (a) a linear
5   vector comprising the chimeric fiber and a wild-type E4
gene, and (b) a linear vector that is E4⁻.
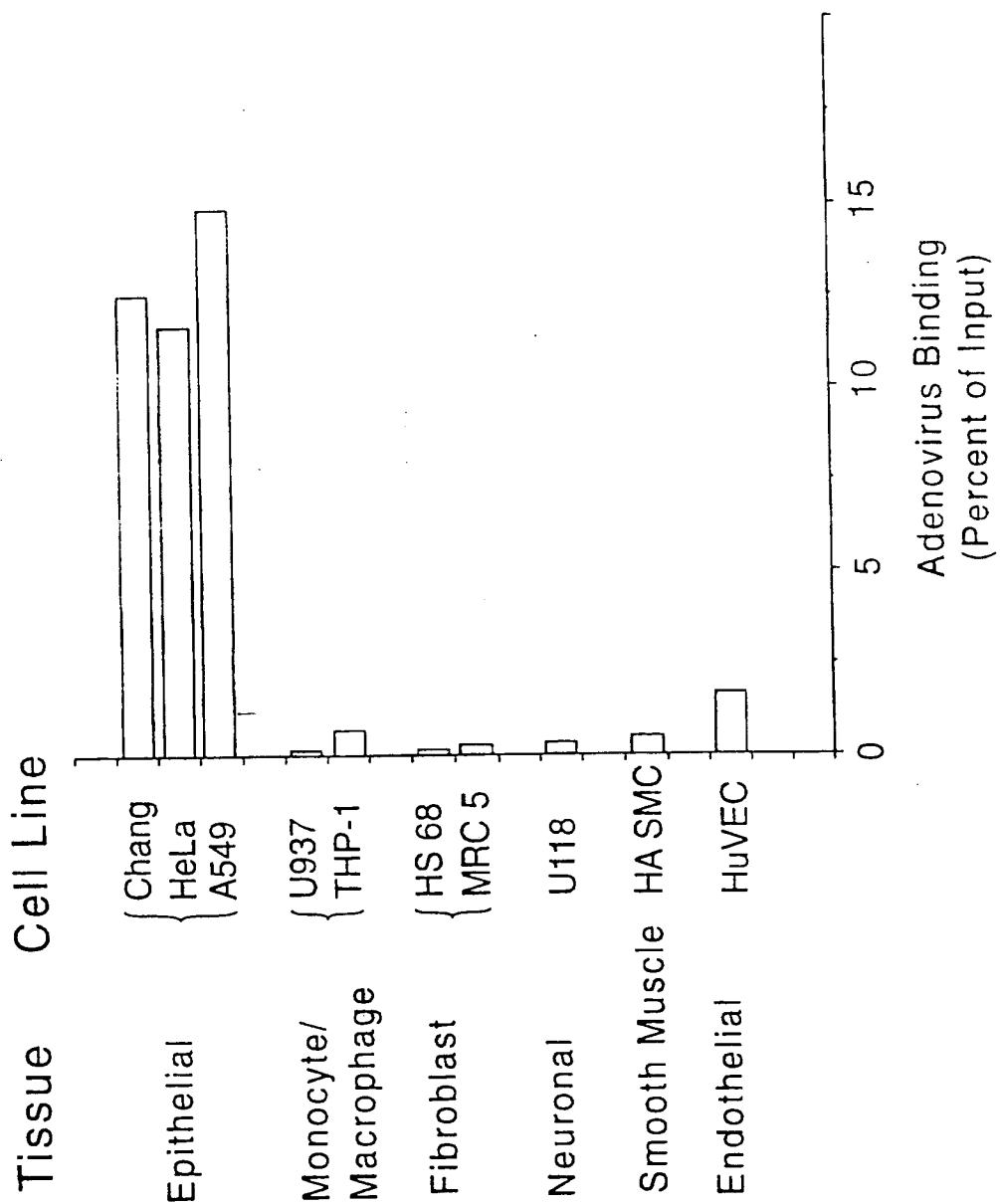
FIG. 1

Ala Gln Glu *

GCC CAA GAA **TAA** AGA ATC GTT TGT GTT ATG TTT CAA CGT   [SEQ ID NO:13]

TRANSCRIPTION

GCC CAA GAA UAA AGA AUC GUU UGU GUU AAA AAA AAA AAA AAA AAA .... [SEQ ID NO:14]

TRANSLATION

Ala Gln Glu *

FIG. 2A

Ala Gln Glu Gly Ser Asn Lys Glu Ser Phe Val Leu Cys [SEQ ID NO:16]

GCC CAA GAA GGA TCC AAT AAA GAA TCG TTT GTG TTA TGT [SEQ ID NO:15]
           BamHI   PolyA

TRANSCRIPTION

GCC CAA GAA GGA UCC AAU AAA GAA UCG UUU GUG UUA AAA AAA AAA AAA AAA...[SEQ ID NO:17]

TRANSLATION

Ala Gln Glu Gly Ser Asn Lys Glu Ser Phe Val Leu Lys Lys Lys Lys Lys...[SEQ ID NO:18]

FIG. 2B

4/30

NdeI   BamHI   MunI

TATGGAGGATCCAATAAAGAATCGTTTGTGTTATGTTTCAACGTGTTTATTTTTC [SEQ ID NO:9]
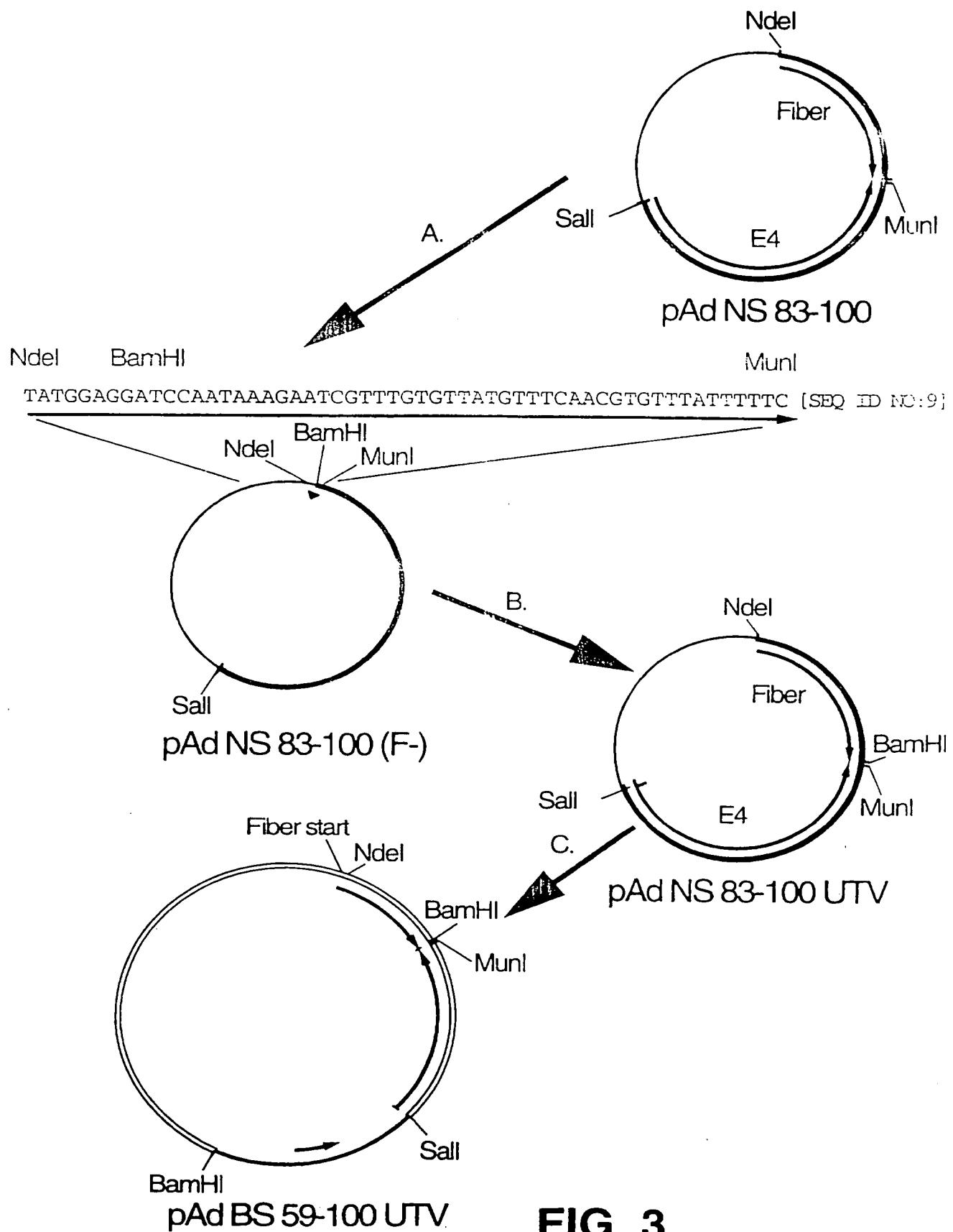
FIG. 3

TAT GGA GGA TCC AAT AAA GAA TCG TTT GTG TTA TGT TTC AAC GTG TTT ATT TTT C [SEQ ID NO:9]

Ndel   BamHI   PolyA   poly A   MunI

FIG. 4A

AAT TGA AAA ATA AAC ACG TTG AAA CAT AAC ACA AAC GAT TCT TTA TTG GAT CCT CCA [SEQ ID NO:10]

MunI   PolyA   BamHI   Ndel

FIG. 4B

TCCC CCCGGG TCTAGA TTA GGA TCC TCC TTG GGC AAT GTA TGA [SEQ ID NO:11]

    XmaI   XbaI   BamHI

FIG. 4C

CGT GTA TCC ATA TGA CAC AGA [SEQ ID NO:12]

           Ndel

FIG. 4D

WT UTV
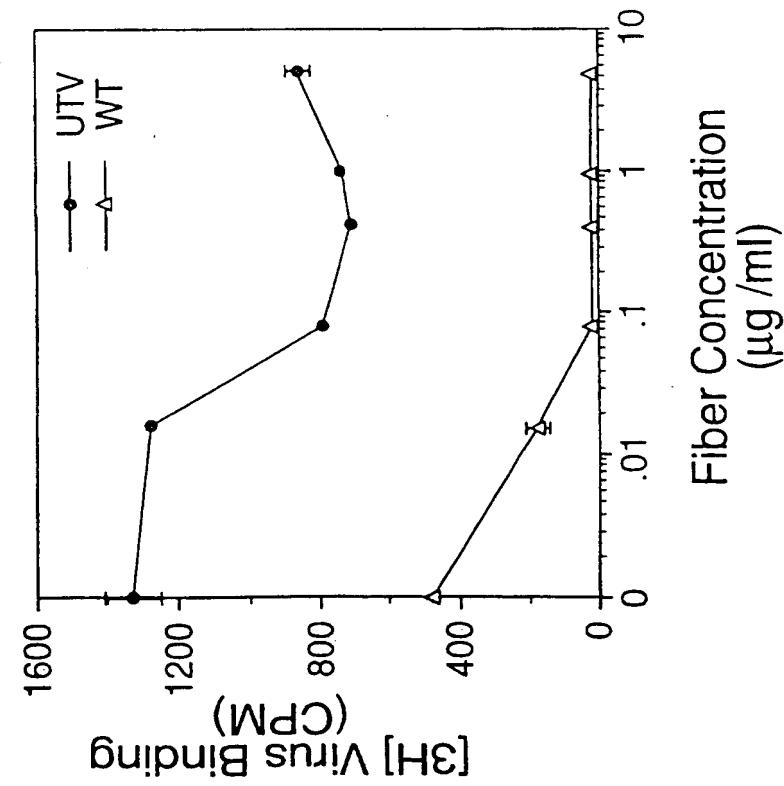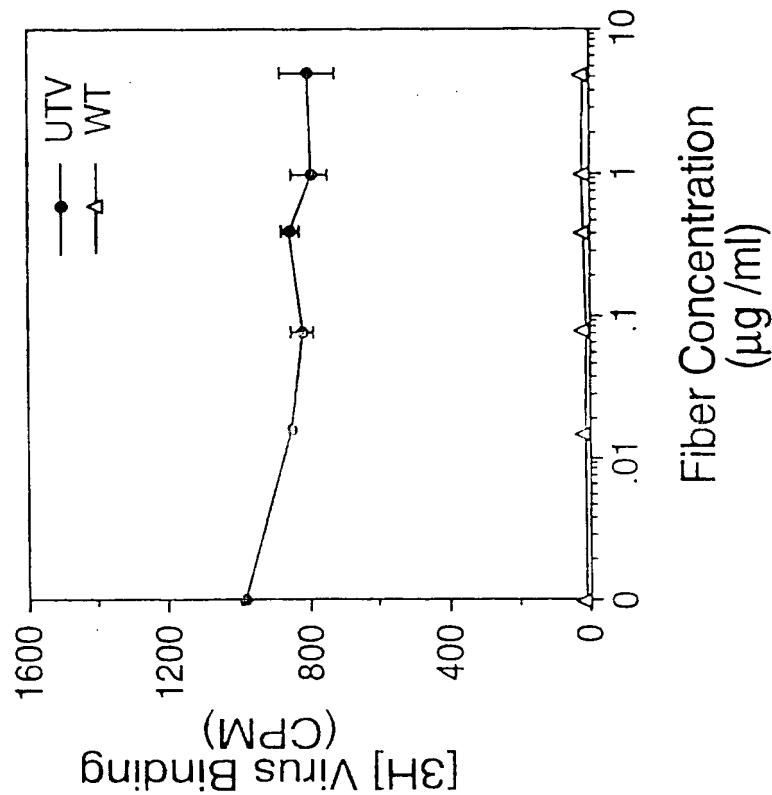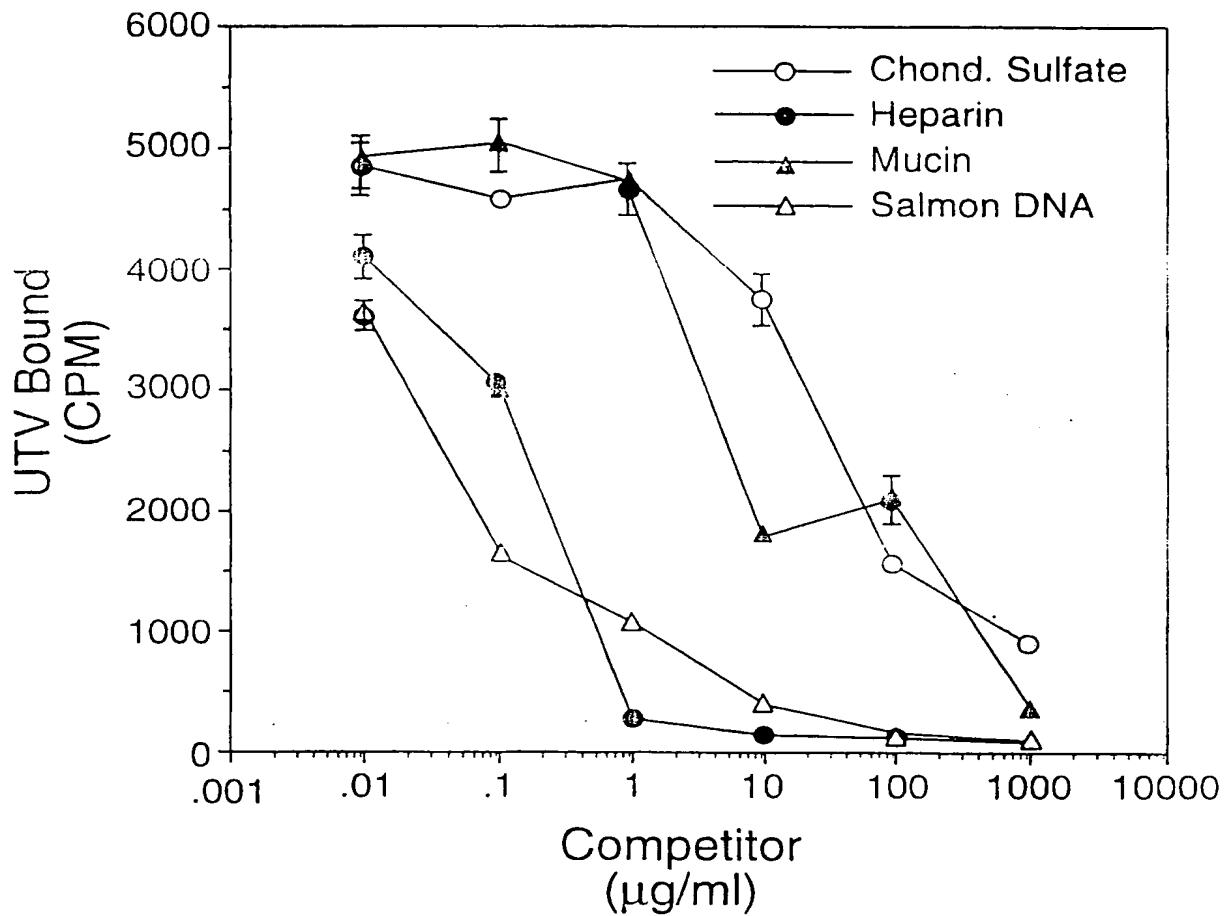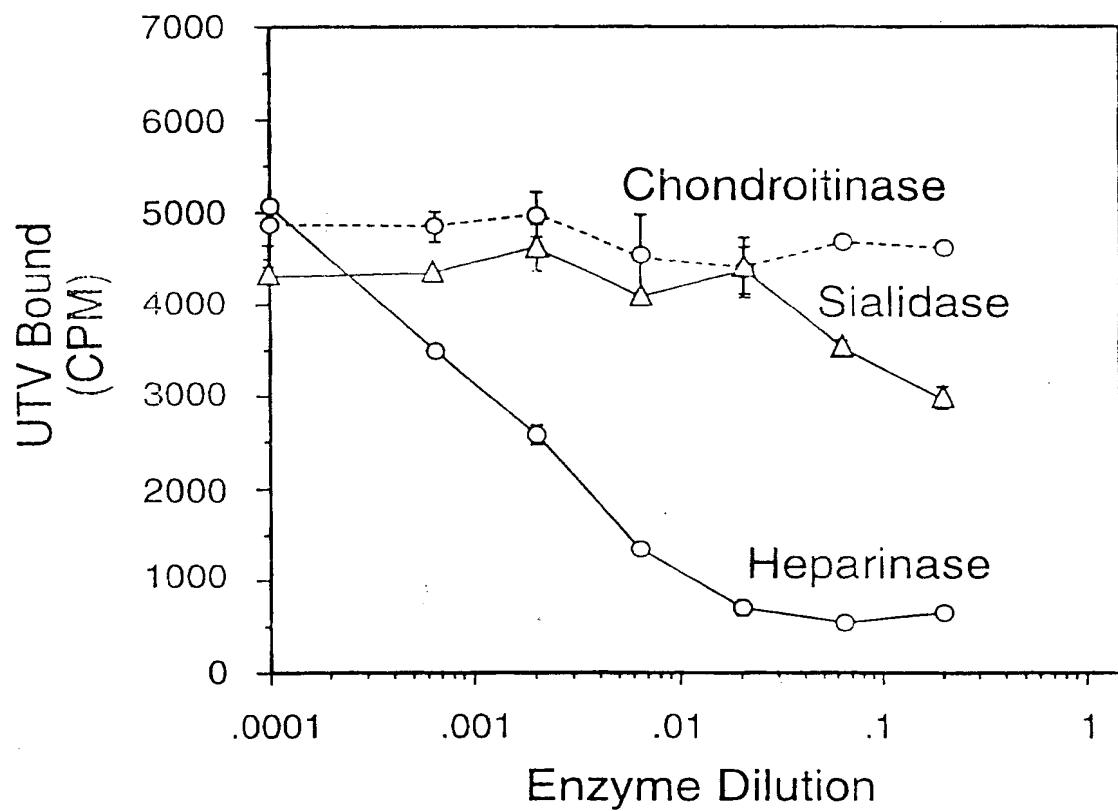
62 kD → ▬ ▬

FIG. 5

FIG. 6B

FIG. 6A

FIG. 7

FIG. 8

FIG. 9

FIG. 10

FIG. 11

BamHI　　polyA

TCA TAC ATT GCC CAA GAA GGA TCC **AATAAA** GAA [SEQ ID NO:19]

S　Y　I　A　Q　E　G　S　　　　　　　　　　[SEQ ID NO:20]

NdeI

Fiber

BamHI

p193 (F5*)
(7293 bp)

ScaI

SalI
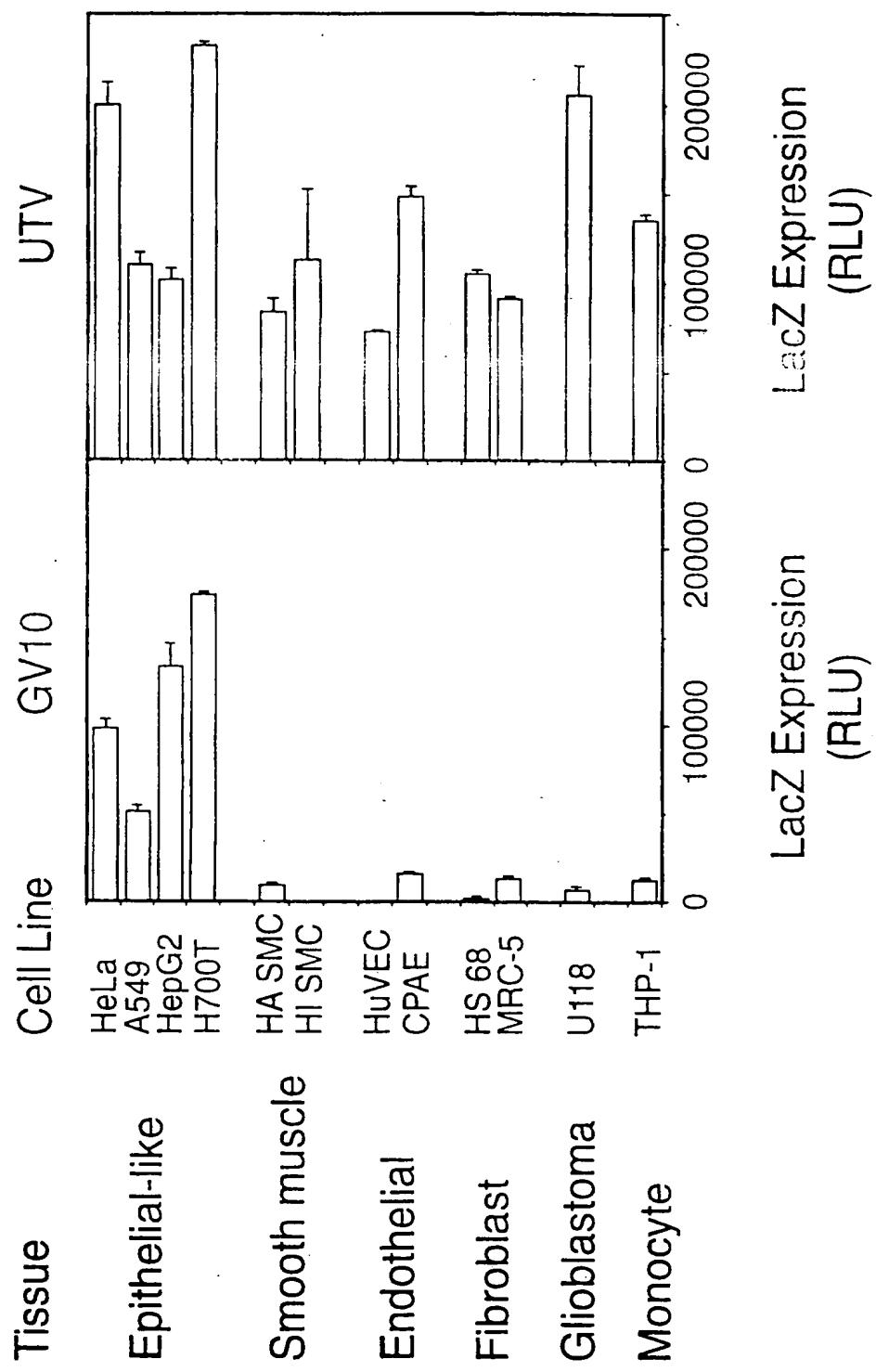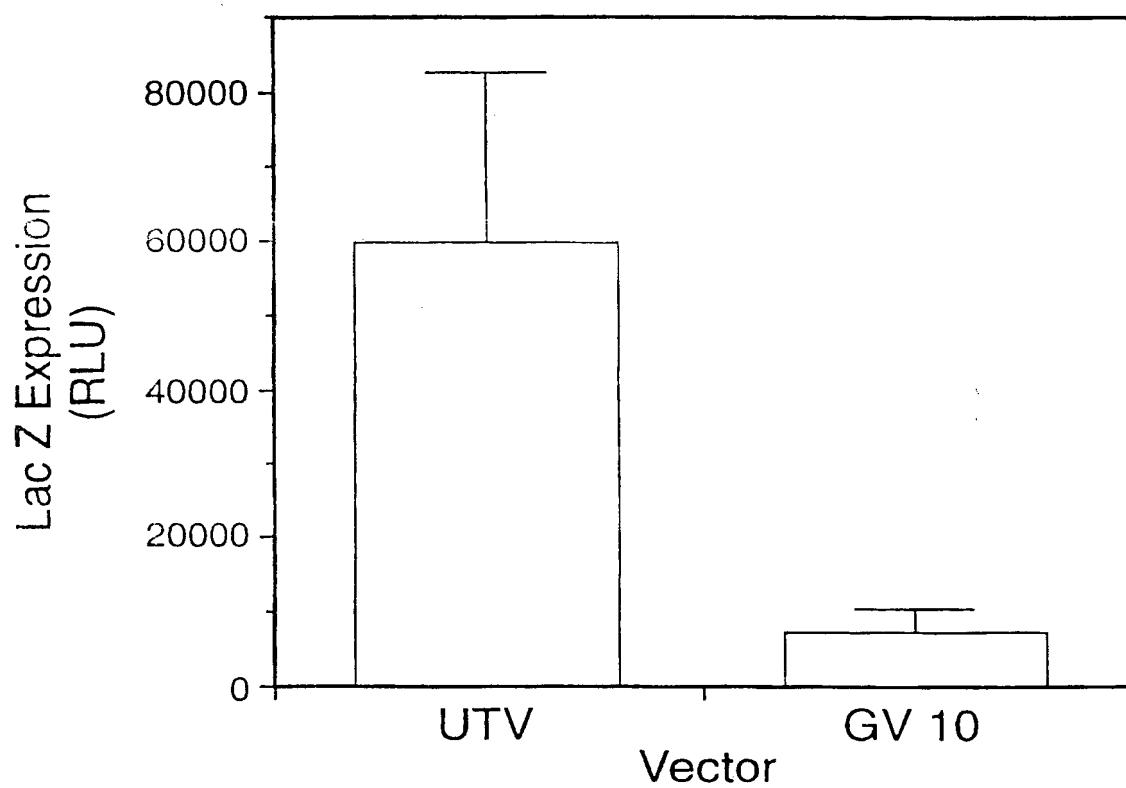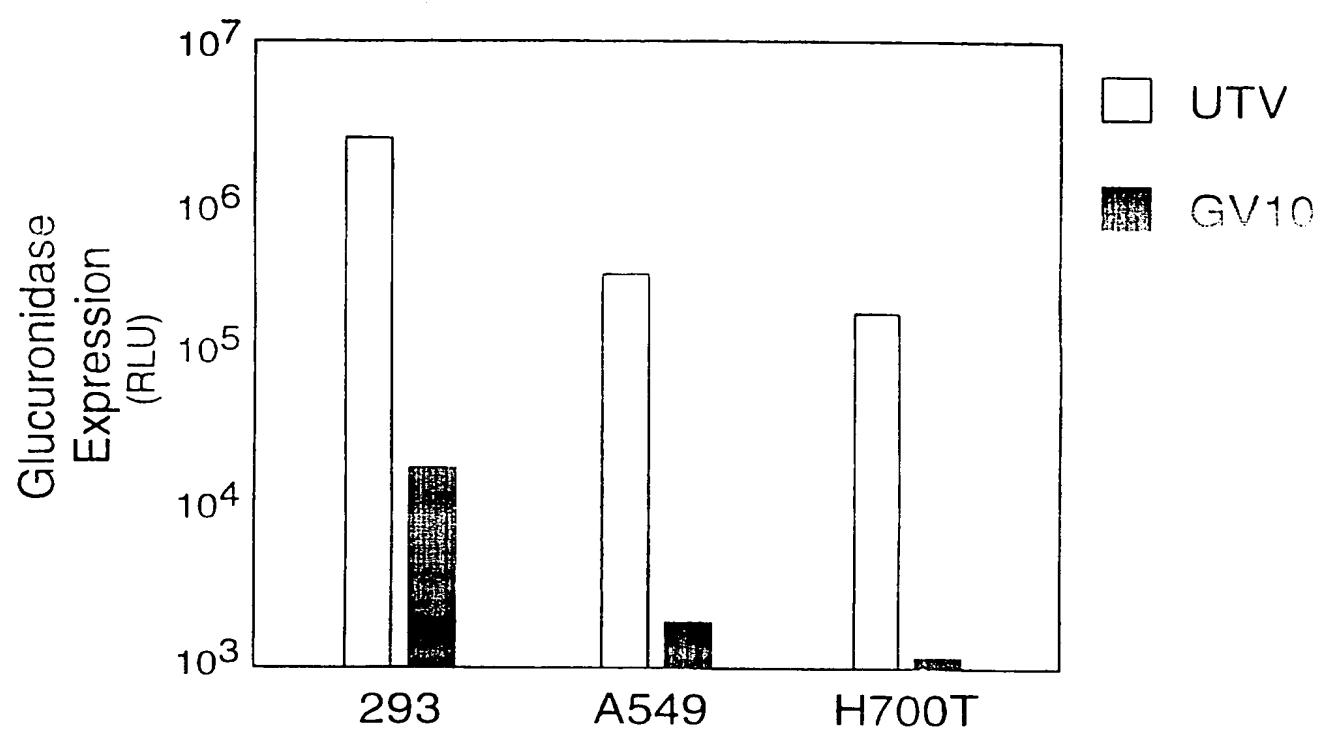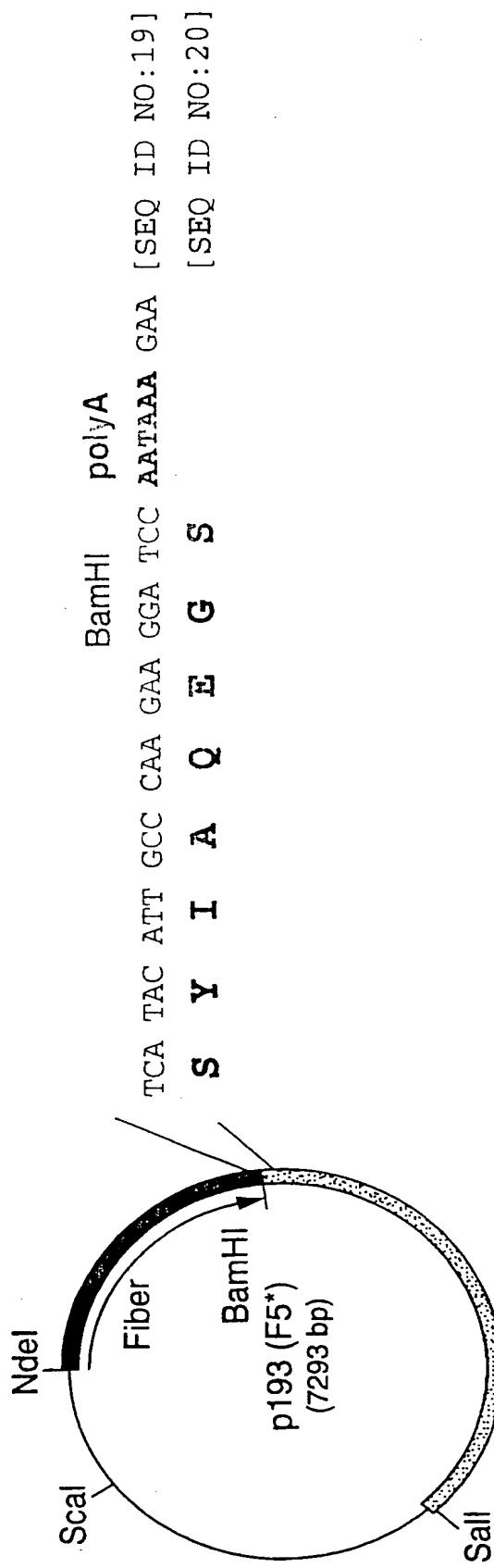
FIG. 12
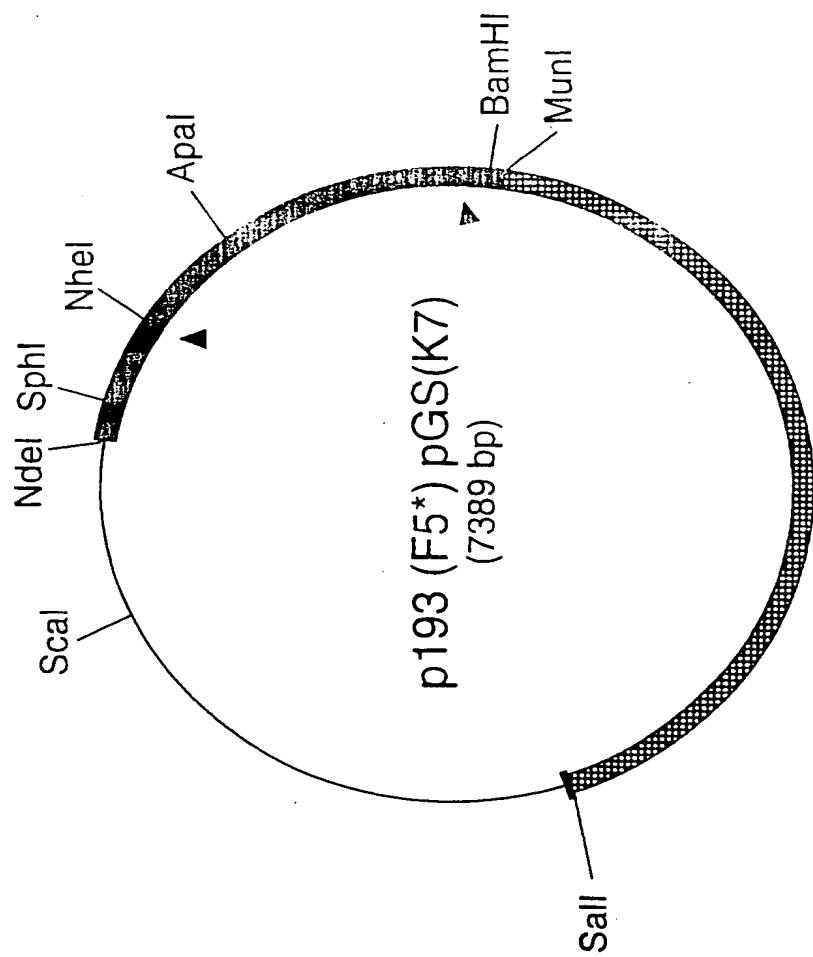
FIG. 13

GGATCAGGATCAGGTTCAGGGAGTGGCTCTAAAAAGAAGAAAAAAGAAGAAGTAA   [SEQ ID NO:21]
GlySerGlySerGlySerGlySerGlySerLysLysLysLysLysLysLys   [SEQ ID NO:22]

pBSS75-100ΔE3 pGS (null)
(9972)
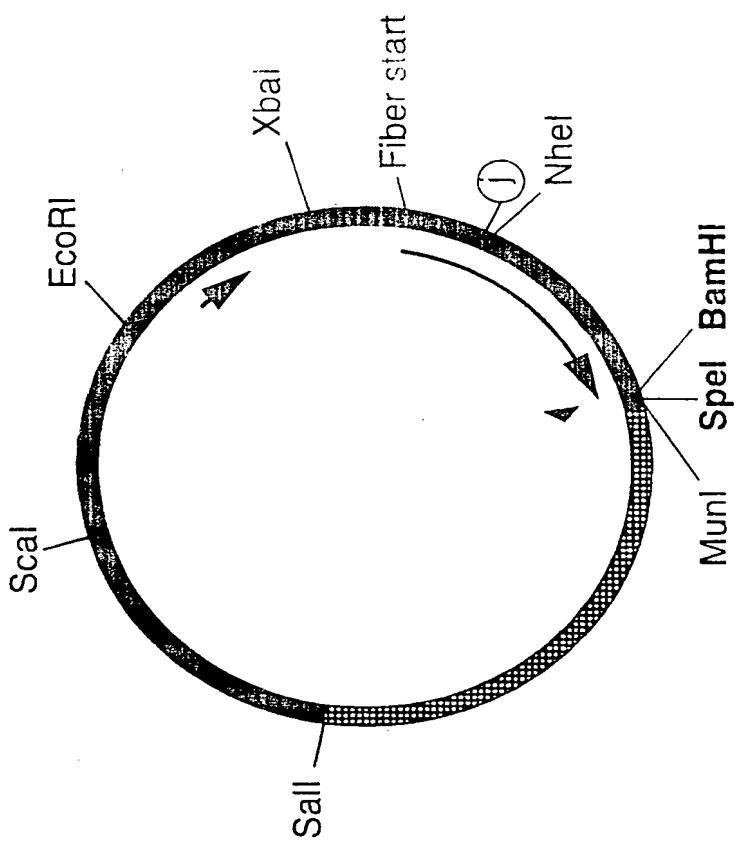
FIG. 14

BamHI
GCCCAAGAAGGATCCGGTTCAGGATCTGGCCAGTGGCTCGACTAGTTAA [SEQ ID NO:23]
AlaGlnGluGlySerGlySerGlySerGlySerGlySerThrSer   [SEQ ID NO:24]

pBSS75-100ΔE3 pGS(RKKK)2
(9999bp)

Scal

EcoRI

Fiber Start

Nhel

Sall

BamHI

MunI Spel

FIG. 15

Spel [SEQ ID NO:25]
[SEQ ID NO:26]

BamHI
GCCCAAGAAGAGGATCCGGTTCAGGATCTGGCCAGTGGCTCGACTAGAAAGAAGAAGAAACGCAAAAAGAAGAAGACTAGTTAA [SEQ ID NO:25]
AlaGlnGluGluGlyGlySerGlySerGlySerGlySerGlySerThrArgLysLysArgLysLysArgLysLysLysThrSer [SEQ ID NO:26]

pBSS75-100ΔE3 pGS(RKKK)3
(10010bp)

FIG. 16

BamHI

GCCCAAGAAGAGGATCCGGTTCAGGAGATCTGGCAGTGGCTCGACTAGAAAGAAGAAGCGCAAAAAAAAGAAGAAGCCAAAAAAAAGAAAAAGAAGACTAGTTAA [SEQ ID NO:27]

AlaGlnGluGlySerGlySerGlySerGlySerGlySerThrArgLysLysLysArgLysLysLysArgLysLysLysArgLysLysThrSer [SEQ ID NO:28]

                                                  SpeI

Spel

ATTACACTTAATGGC**ACTAGT**GAATCCACAGAAACT   [SEQ ID NO:29]
IleThrLeuAsnGlyThrSerGluSerThrGluThr   [SEQ ID NO:30]

FIG. 17
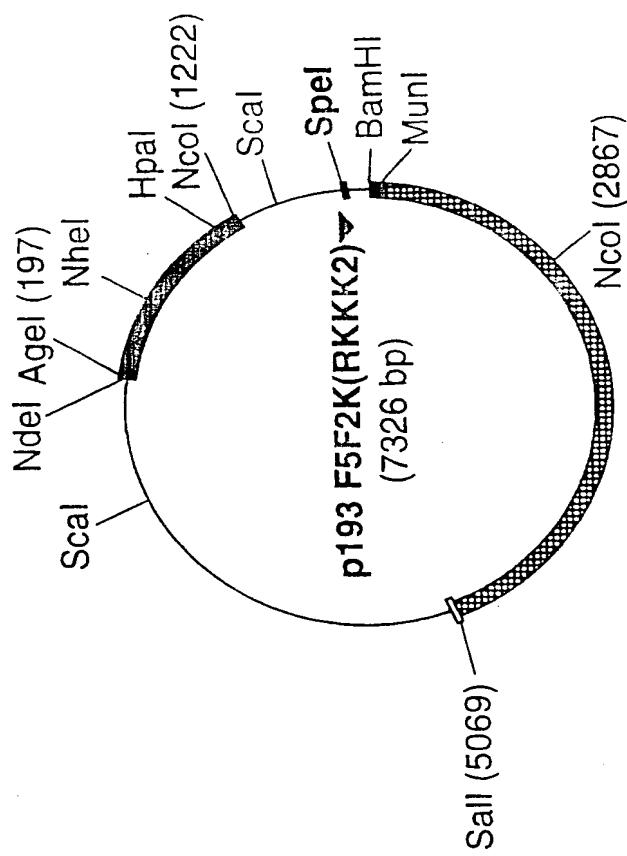
FIG. 18

ATTACACTTAATGGCACTAGAAAGAAGAAACGCAAAAGAAGAAGACTAGTGAATCCACAGAAACT [SEQ ID NO:31]
IleThrLeuAsnGlyThrArgLysLysLysArgLysLysLysThrSerGluSerThrGluThr [SEQ ID NO:32]

Spel

FIG. 19

Spel

CTTAATGGCACTAGAAGAAGAGCGCAAAAAAAAGAAGAAGAAGAAGACTAGTGAATCCACA   [SEQ ID NO:33]
LeuAsnGlyThrArgLysLysLysLysArgLysLysLysLysLysLysLysLysLysThrSerGluSerThr   [SEQ ID NO:34]

Pmel

BstXl
EcoRl
PB start

Ascl
BamHl

pACT (RK33)
(11007 bp)

Ascl Spel
PB stop

Bglll
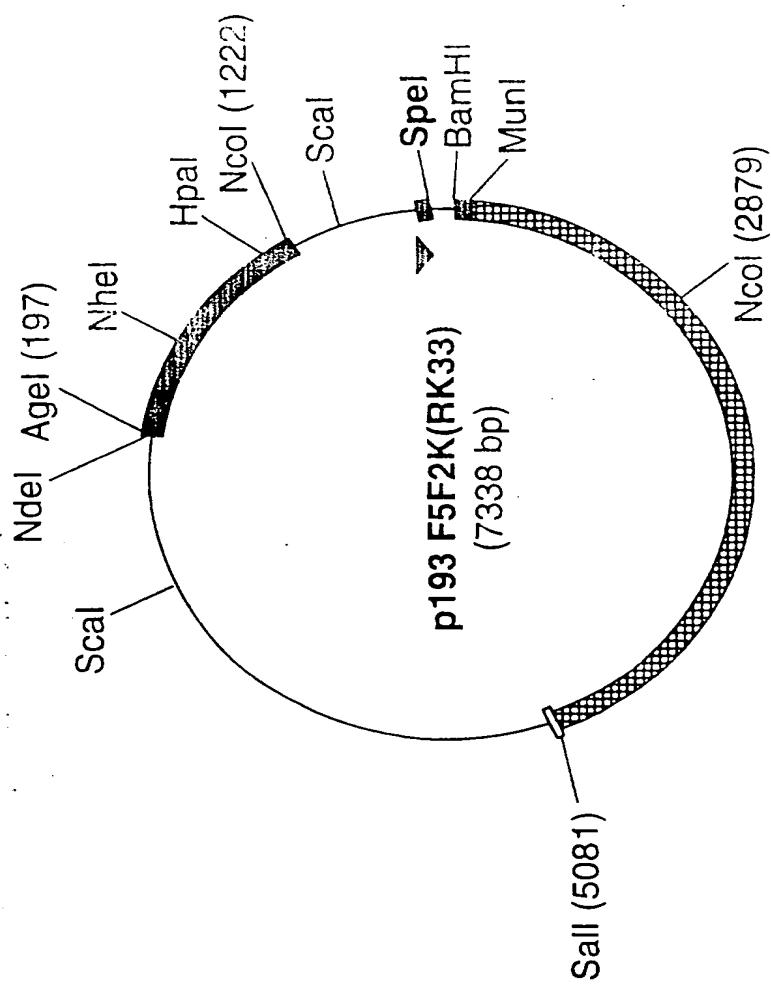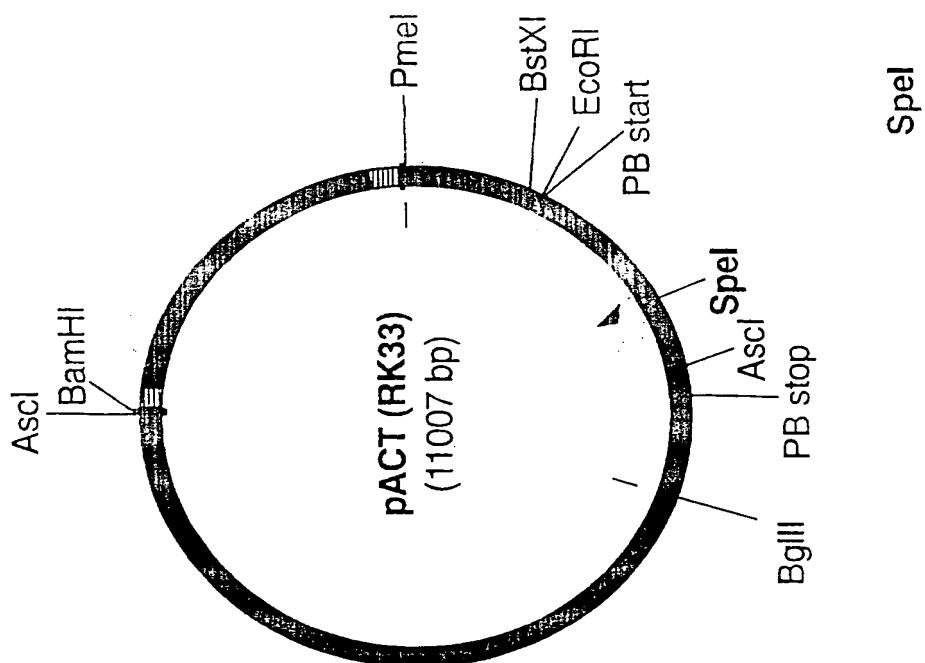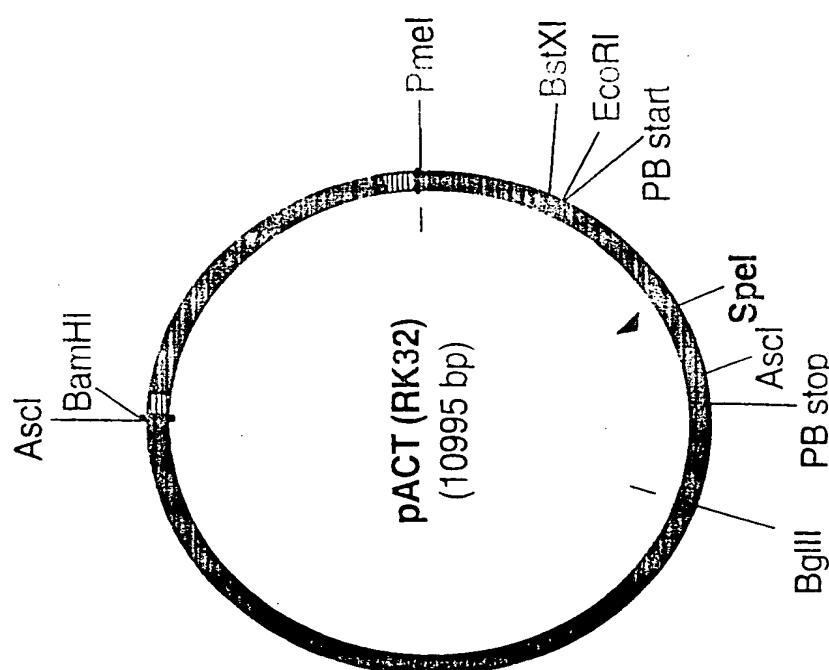
Spel

AACGATACTAGAAAGAAGCGCAAAAAAGAAGAAGAAGACTAGTGCCACA   [SEQ ID NO:35]
AsnAspThrArgLysLysArgLysLysArgLysLysLysArgLysLysLysThrSerAlaThr   [SEQ ID NO:36]

FIG. 20

FIG. 2

AACGATACTAGAAAGAGAAAGAGAAAGAGAAAGAGACTAGTGCCACA [SEQ ID NO:37]
AsnAspThrArgLysLysArgLysLysArgLysLysThrSerAlaThr [SEQ ID NO:38]
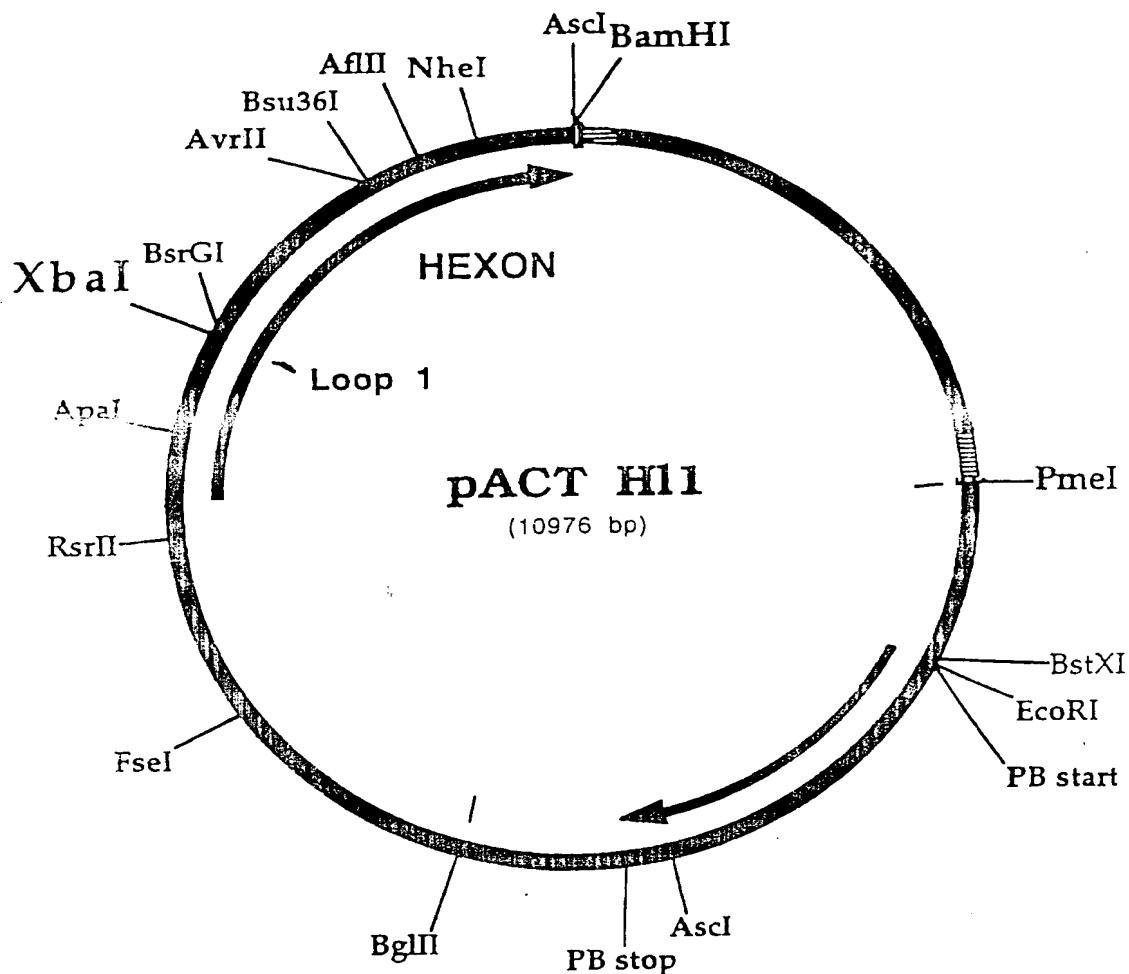
SpeI

XbaI

ACCGCA TCTAGA GGTGATAACTTG [SEQ ID NO: 39]
ThrAlaSerArgGlyAspAsnLeu [SEQ ID NO: 40]

Figure 22
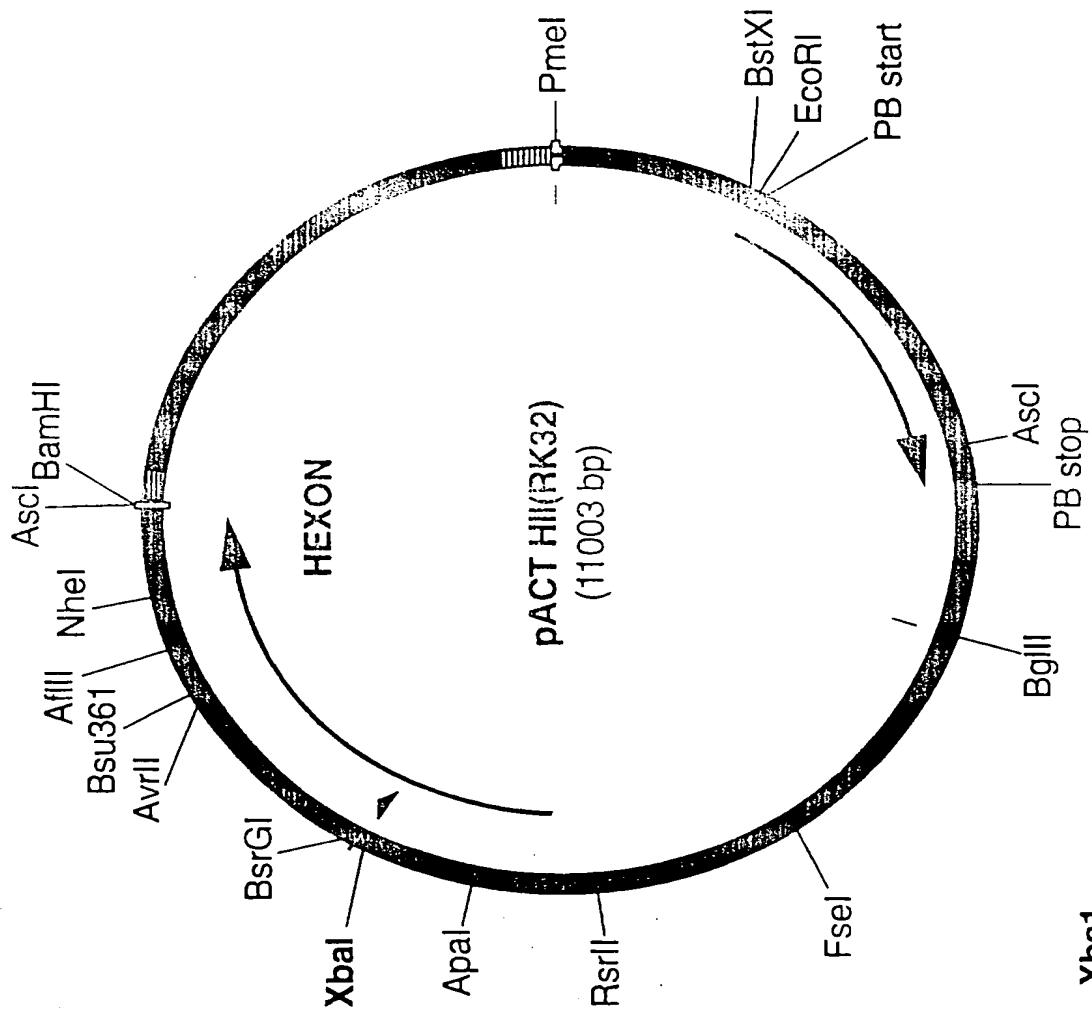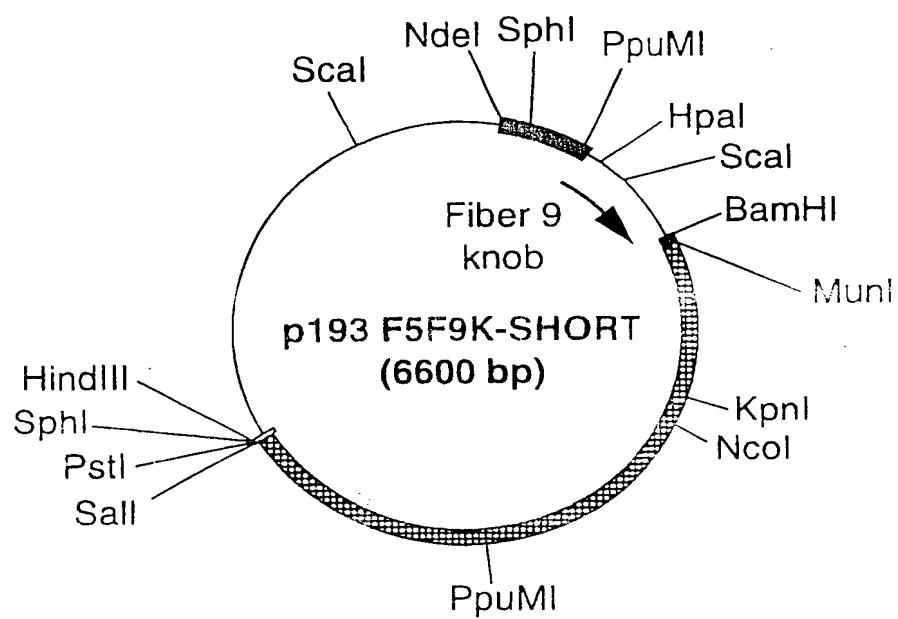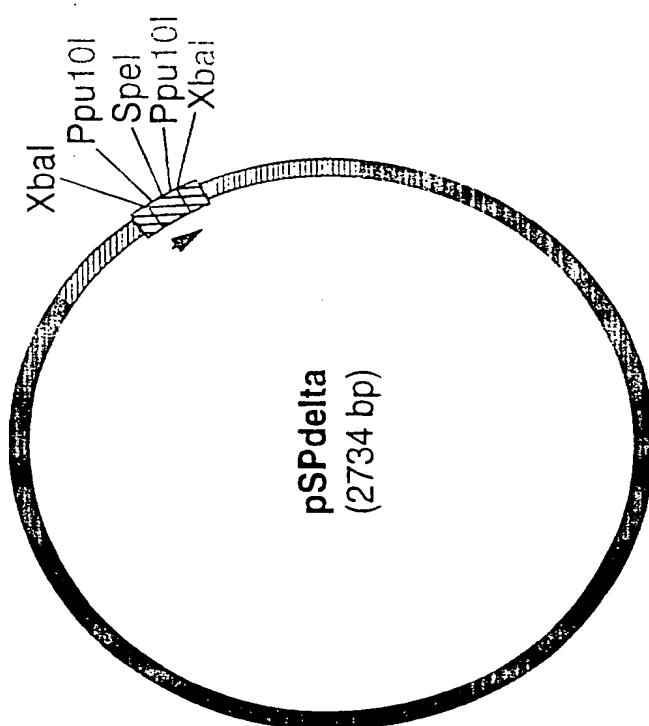
FIG. 23

Xba1

ACCGCATCTAGAAAGAAGAAACGCAAAAGAAGACTAGAGGTGATAACTTG [SEQ ID NO:41]

ThrAlaSerArgLysLysLysArgLysLysLysThrArgGlyAspAsnLeu [SEQ ID NO:42]

FIG. 24

Xbal   Ppu10I   Spel   Ppu10I   Xbal

TCTAGAGCAGCTATGCATGAAGGACTAGTGGAGAGATGCATGCAGCCTCTAGA [SEQ ID NO:43]
SerArgAlaAlaMetHisGluGlyThrSerGlyGluMetHisAlaAlaSerArg [SEQ ID NO:44]

FIG. 25

XbaI   (j)

TCTAGAGCAGCTATGCAGGCGGCCAGAAGCTGAAGAGGCCAGCCACACGGGCTGAGGAGAAGCGCGCCTGAGGCCGAAG
SerArgAlaAlaMetGlnAlaAlaGluAlaGluGluLysArgAlaGluGluLysArgAlaGluAlaGluA

CAGCGGGCCGAAGCTGCCGCCCCCGCTGCCAACCCGAGGTCGCGAGAAGCCTCGAGAAGAAACAGGCTAAGCGCCGA
laAlaAlaGluAlaAlaProAlaAlaAlaGlnProGluValGluLysProGlnLysLysGlnAlaLysAlaAlaGl

AGCTGTGCAGGCGGCCCGGCCCGCAGAAGCTGAAGAGGCCAGCCACACGGGCTGAGGAGAAGCGCGCCTGAGGCCAGCCAGCGGGCC
uAlaValGlnAlaAlaAlaGluAlaGluGluLysArgAlaGluGluLysArgAlaGluAlaGluAlaAla

                     (j)

GAAGCTGCCGCCCCCGCTGCCAACCCGAGGTCGCGAGAAGCCTCGAGAAGAAACAGGCTAAGCGCTAAGCGCTGTGC
GluAlaAlaAlaProAlaAlaAlaGlnProGluValGluLysProGlnLysLysGlnAlaLysAlaAlaGluAlaValH
  SpeI   Ppu10I   XbaI

ATGAAGGGACTAGTGGAGAGATGCATGCAGCCTCTAGA   [SEQ ID NO: 45]
isGluGlyThrSerGlyGluMetHisAlaAlaSerArg   [SEQ ID NO: 46]

FIG. 26

pSP2alpha2
(3334 bp)
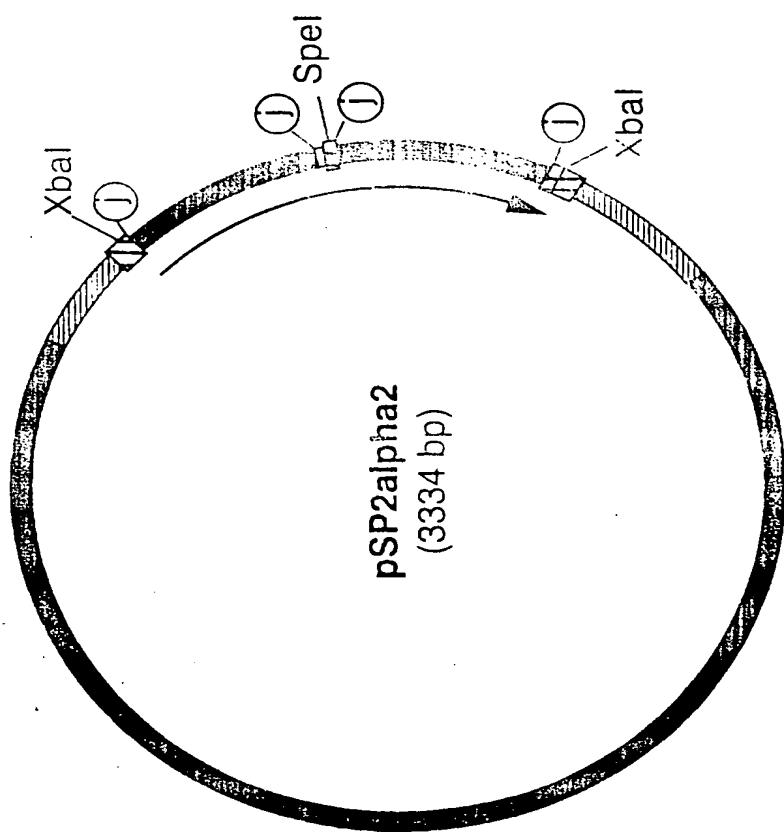
FIG. 27

XbaI   ⓙ
TCT AGA GCA GCT ATG CAG GCG GCC GCA GAA GCT GAA GAG GCA GCC
Ser Arg Ala Ala Met Gln Ala Ala Ala Glu Ala Glu Glu Ala Ala
ACA CGG GCT GAG GAG AAG CGC GCT GAG GCC GAA GCA GCG GCC GAA
Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala Glu Ala Ala Ala Glu
GCT GCC GCC CCC GCT GCC CAA CCC GAG GTC GAG AAG CCT CAG AAG
Ala Ala Ala Pro Ala Ala Gln Pro Glu Val Glu Lys Pro Gln Lys
AAA CAG GCT AAC GCT AAG GCC GAA GCT GTG CAG GCG GCC GCA GAA
Lys Gln Ala Asn Ala Lys Ala Glu Ala Val Gln Ala Ala Ala Glu
GCT GAA GAG GCA GCC ACA CGG GCT GAG GAG AAG CGC GCT GAG GCC
Ala Glu Glu Ala Ala Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala
GAA GCA GCG GCC GAA GCT GCC GCC CCC GCT GCG CAA CCC GAG GTC
Glu Ala Ala Ala Glu Ala Ala Ala Pro Ala Ala Gln Pro Glu Val
                                                          ⓙ
GAG AAG CCT CAG AAG AAA CAG GCT AAC GCT AAG GCC GAA GCT GTG
Glu Lys Pro Gln Lys Lys Gln Ala Asn Ala Lys Ala Glu Ala Val
        SpeI   ⓙ
CAT GAA GGG ACT AGT GGA GAG ATG CAG GCG GCC GCA GAA GCT GAA
His Glu Gly Thr Ser Gly Glu Met Gln Ala Ala Ala Glu Ala Glu
GAG GCA GCC ACA CGG GCT GAG GAG AAG CGC GCT GAG GCC GAA GCA
Glu Ala Ala Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala Glu Ala
GCG GCC GAA GCT GCC GCC CCC GCT GCG CAA CCC GAG GTC GAG AAG
Ala Ala Glu Ala Ala Ala Pro Ala Ala Gln Pro Glu Val Glu Lys
CCT CAG AAG AAA CAG GCT AAC GCT AAG GCC GAA GCT GTG CAG GCG
Pro Gin Lys Lys Gln Ala Asn Ala Lys Ala Glu Ala Val Gln Ala
GCC GCA GAA GCT GAA GAG GCA GCC ACA CGG GCT GAC GAG AAG CGC
Ala Ala Glu Ala Glu Glu Ala Ala Thr Arg Ala Glu Glu Lys Arg
GCT GAG GCC GAA GCA GCG GCC GAA GCT GCC GCC CCC GCT GCG CAA
Ala Glu Ala Glu Ala Ala Ala Glu Ala Ala Ala Pro Ala Ala Gln
CCC GAG GTC GAG AAG CCT CAG AAG AAA CAG GCT AAC GCT AAG GCC
Pro Glu Val Glu Lys Pro Gln Lys Lys Gln Ala Asn Ala Lys Ala
        ⓙ   **XbaI**
GAA GCT GTG CAT GCA GCC TCT AGA   [SEQ ID NO:47]
Glu Ala Val His Ala Ala Ser Arg   [SEQ ID NO:48]

FIG. 27 Cont.

FIG. 28